HO-1 induction by Co-PPIX suppresses experimental skin inflammation, T cell immunity and dendritic cell maturation and function

Dissertation

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Der Beginn aller Wissenschaft ist das Erstaunen,
dass die Dinge so sind, wie sie sind.
Aristoteles

Allen, die ich im Herzen trage

Zusammenfassung

Die Hämoxygenase 1 (HO-1) ist ein Stressprotein mit antientzündlichen, immunsupprimierenden und zytoprotektiven Eigenschaften, welche in vielen Tiermodellen nachgewiesen wurden. Die zugrunde liegenden Mechanismen sind wenig bekannt.

Diese Arbeit demonstriert erstmalig, dass die physiologische Induktion von HO-1 wichtig für die Limitierung von T-Zell-abhängigen Hautentzündungen ist. So führt der HO-1-Inhibitor, Zinn-Protoporphyrin IX (Sn-PPIX), zu einer verstärkten Hautentzündung im Mausmodell. Die pharmakologische Induktion von HO-1 durch Kobalt-Protoporphyrin IX, Co-PPIX, hemmt dagegen die Entzündung in DNFB- bzw. TMA-induzierten murinen Kontaktallergiemodellen sowohl bei Verabreichung von Co-PPIX während der Sensibilisierung als auch vor der Auslösung.

Bemerkenswerterweise hemmt eine Co-PPIX-Behandlung die Antigen-induzierte T-Zellproliferation *ex vivo* in Milzzellen von behandelten Mäusen und *in vitro* in humanen mononukleären Zellen des peripheren Blutes. Da eine HO-1-Induktion durch Co-PPIX nur in Monozyten und in aus Monozyten abgeleiteten myloischen Dendritischen Zellen (MDDC), nicht aber in T-Zellen, beobachtet wurde, fokussierten alle weiteren Untersuchungen auf Antigen-präsentierende Zellen.

HO-1-Induktion durch Co-PPIX reduziert die Expression von MHC-Klasse II und akzessorischen Molekülen und steigert die Phagozytose und den oxidativen Burst von Monozyten. Die immunphänotypische Differenzierung und Maturierung von MDDC wird gehemmt. Funktionsteste zeigen eine Reduktion der Expression und Sekretion von proinflammatorischen und immunstimulatorischen Zytokinen, während die Sekretion des antientzündlichen Zytokins IL-10 gesteigert ist. Die Fähigkeit der MDDC zur Antigenpräsentation gegenüber T-Helferzellen ist für Allo- und Recallantigene stark herabgesetzt. Mittels adenoviraler HO-1-Transduktion von MDDC konnte die Spezifität der Effekte bestätigt werden.

Diese Daten zeigen, dass eine verstärkte HO-1-Aktivität die Dendritischen Zellen zu einem unreifen und immunkompromittierten Phänotyp verändert und weisen darauf hin, dass die HO-1-Induktion einen wichtigen Ansatz für die Hemmung der zellulären Immunität und für die Behandlung von T-Zell-abhängigen Hautentzündungen darstellt.

Abstract

Heme oxygenase 1 (HO-1) is an antiinflammatory stress protein. Its immunosuppressive and cytoprotective activities have been demonstrated in several animal models. The underlying mechanisms, however, are poorly understood.

This study demonstrates for the first time that the physiological induction of HO-1 is important for the limitation and resolution of T cell-dependent skin inflammation. So, the HO-1 inhibitor, tin protoporphyrin IX (Sn-PPIX), augments cutaneous inflammation in mouse model. Moreover, pharmacologic HO-1 induction by the potent HO-1 inducer, cobaltic protoporphyrin IX (Co-PPIX), inhibits inflammation when applied around sensitization or before challenge in murine DNFB- and TMA-induced contact hypersensitivity models.

Remarkably, Co-PPIX treatment inhibits antigen-driven T cell proliferation both *ex vivo* in murine splenocytes and *in vitro* in human peripheral blood mononuclear cells. Since induction of HO-1 mRNA and protein was found in monocytes and monocyte-derived myeloid dendritic cells (MDDC) but not T cells, further investigations focused on antigen-presenting cells. HO-1 induction by Co-PPIX depresses monocytic MHC class II and accessory molecule expression whereas phagocytosis and respiratory burst activities are augmented. Moreover, HO-1 induction inhibits the immunophenotypic differentiation and maturation of MDDC. Functional analysis revealed a decreased proinflammatory cytokine production whereas secretion of the antiinflammatory cytokine IL-10 is increased. Remarkably, the antigen-presenting capacity of MDDC for T-helper cells is diminished both for allo- and for recall-antigens. Adenoviral HO-1 transduction of MDDC confirmed that the effects are mediated by HO-1.

These data indicate that an enhanced HO-1 activity switches myeloid DCs to an immature and functionally compromised phenotype and suggest that HO-1 induction represents an important approach for depressing T cell immunity and for the treatment of T cell-dependent skin inflammation.

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Abbreviations

µg	microgram
µL	microliter
µmol	micromol
Ab	antibody
Ad	adenovirus
Ag	antigen
APC	antigen-presenting cell
BCA	bicinchoninic acid
Bidest	distillated water
BSA	bovine serum albumin
CD	cluster of differentiation
CFDA-SE	carboxyfluorescein diacetate succinimidyl ester
cGMP	cyclic guanosine monophosphate
CHS	contact hypersensitivity
CO	carbon monoxide
Co-PPIX	cobaltic protoporphyrine IX
DC	dendritic cell
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNFB	dinitrofluorobenzene
DTH	delayed-type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
E. coli	Escherichia coli
EDTA	ethylendiamin tetraacid
e.g.	example gracia
ELISA	enzyme linked immunosorbent assay
et al	et alii
FACS	fluorescence activated cell scanner
Fc	fragment crystalline
FCS	fetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanat
FL	fluorescence
g	relative centrifugal force

GeoMean	geometric mean
GM-CSF	granulocyte/macrophage colony-stimulating factor
h	human
HE	Hematoxylin-Eosin
HLA	human leukocyte antigen
HO	heme oxygenase
hPBMC	human peripheral blood mononuclear cell
HSP	heat shock protein
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iMDDC	immature monocyte-derived dendritic ells
iNOS	inducible nitric oxide synthase
kDa	kilodalton
kg	kilogram
L	liter
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
LTT	lymphocyte transformation test
M	molar
MACS	magnetic cell sorting
M-CSF	monocyte/macrophage colony-stimulating factor
MDDC	monocyte-derived dendritic cells
MFI	mean fluorescence intensity
mg	milligram
MHC	major histocompatibility complex
min	minute
MLR	mixed leukocyte reaction
mM	millimolar
mMDDC	mature monocyte-derived dendritic cells
NADPH	nicotinamide adenine dinucleotide phosphate
NFAT	nuclear factor of activated T cells
NFkB	nuclear factor-kappaB
NK	natural killer
NMRI	Naval Medical Research Institute
NO	nitric oxide

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
Pi	propidium iodide
PS	phosphatidylserine
R	receptor
rpm	rounds per minute
RNA	ribonucleic acid
RT	room temperature
SD	standard deviation
SEM	standard error of mean
Sn-PPIX	tin protoporphyrine IX
STAT	signal transducer and activator of transcription
TAC	tetrameric antibody complex
TCR	T cell receptor
TGF	transforming growth factor
Th	T-helper
TLR	Toll-like receptors
TMA	trimellitic anhydride
TNF-α	tumor necrosis factor α
UV	ultraviolet
VSMC	vascular smooth muscle cells
v/v	volume per volume
WB	Western blot
w/v	weight per volume

1 Introduction

1.1 Heme oxygenase 1 increase as a cytoprotective and antiinflammatory principle

1.1.1 Heme oxygenases

Heme oxygenase activity was discovered in the late 1960s by Rudi Schmid and colleagues (Tenhunen et al, 1968 and 1969). They detected in the microsomal fractions of rat spleen, liver and kidney an enzyme activity that catalyzes the oxidative degradation of heme to biliverdin IXα, carbon monoxide and iron. Later, the HO-1 family arose to three HO isoenzymes (Maines et al, 1986; McCoubrey et al, 1997), whose function consists in the binding and catalytic degradation of the pro-oxidative heme group, that is released by degradation of e.g. hemoglobin, myoglobin, guanylyl cyclase, cyclooxygenase, cytochrome p450 oxidase, inducible nitric oxide synthase (NOS2, iNOS), NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, and also transcription factors Bach 1 and 2 (to which heme can potentially bind) (Otterbein et al, 2003).

HO isoenzymes are different in gene product, gene expression distribution, molecular and biochemical properties and in their own regulation. HO-2 and HO-3 are constitutively expressed in e.g. brain and testes and they show a 90% homology in amino acids. In contrast to the inducible HO-1, the antiinflammatory capacity of HO-2 and HO-3 is low (Shibahara, 2003).

1.1.1.1 Catalysis of heme

The free heme group is a proinflammatory stimulus with effects on lipid peroxidation, vasopermeabilization, leukocyte migration, thrombocyte aggregation, induction of adhesion molecules, protein denaturation and development of free radicals. Consequently cell injury and inflammation develop (Vincent, 1989; Wagener et al, 2001).

Heme oxygenases catalyze the oxidation of the proinflammatory heme group into ferrous iron, carbon monoxide and water-soluble biliverdin. Biliverdin is subsequently reduced to bilirubin by the dual cofactor/pH-dependent soluble biliverdin reductase (Figure 1).

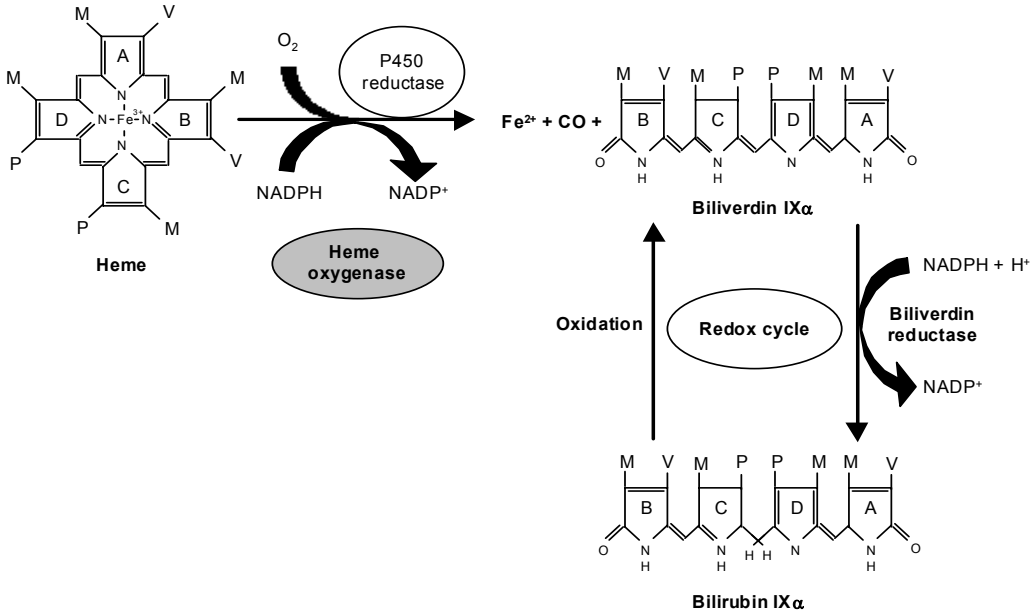


Figure 1. Catalysis of heme by heme oxygenases
Heme oxygenases degrade heme into Fe2+, CO and biliverdin / bilirubin. The reaction requires the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH), molecular oxygen and NADPH-cytochrome P450 reductase.

Bilirubin is an anti-oxidative lipophilic linear tetrapyrrole and is abundant in blood serum. In human, the daily production of bilirubin IXα is between 250 and 400 mg, and about 80% of bilirubin IXα generation accounts to the degradation of hemoglobin (Shibahara, 2003). Bilirubin IXα exhibits hydrophobic properties due to the formation of intramolecular hydrogen bounds. Therefore it must be glucuronidated before excretion in the bile. The catalysis from biliverdin to bilirubin occurs uniquely in mammals (Shibahara, 2003).

The heme degradation products bilirubin and carbon monoxide have antiinflammatory, anti-oxidative, anti-proliferative and anti-apoptotic properties (see 1.1.2.1).

1.1.1.2 Heme oxygenase1 (HO-1)

HO-1 (synonym heat shock protein 32 – HSP32) is, in contrast to HO-2 and HO-3, an inducible "heat shock protein" and has a high catalytic activity in oxygenation of the heme ring. HO-1 is inducible by its substrate heme, by cellular stress (heat shock, oxidative stress, inflammatory stress, UV irradiation, hypoxia, hyperoxia, ischemia, endotoxin, cytokines) and is up-regulated at the transcriptional level by metalloporphyrins, metals, progesterone, curcumin, and IL-10 (Otterbein et al, 2003).

For the antiinflammatory cytokine IL-10, HO-1 induction and mediation of the antiinflammatory effects on mouse macrophages by HO-1 have been reported (Lee et al, 2002). For several plant extracts (caffeic acid and curcumin) HO-1 induction is published, which mediates the known antiinflammatory effects (Motterlini et al, 2000).

The anti-proliferative effect of rapamycin on smooth muscle cells seems to be also HO-1-dependent (Visner et al, 2003). Metalloporphyrins like cobalt (III) protoporphyrin IX (Co-PPIX) have been demonstrated to induce HO-1 expression *in vivo* and *in vitro* (see 1.1.2.5).

1.1.2 Enhanced HO-1 activity as an antiinflammatory principle

1.1.2.1 Molecular mechanism of the antiinflammatory effects of HO-1

The induction of HO-1 ensues by various transcription factors e.g. STAT1, 3 and 5, AP-1, NFkB, Nrf2 and HIF-1, which are activated by heme, iron, inflammatory stimuli and other stressful factors in different cells (Alam et al, 2003; Elbirt et al, 1999; Lee et al, 2000; Li et al, 2001; Ryter et al, 2002 Shibahara, 2003).
The degradation of heme by HO-1 results in depletion of the cellular stressor heme / heme protein and consequently, as feedback regulation, in suppression of iNOS and nitric oxide (NO). HO-1 induces the synthesis of ferritin, resulting in reduction of free iron and decrease in iron-generated reactive O_2 derivatives (Jenkins et al, 1993; Maines MD 1984). Important antiinflammatory and cytoprotective effects of HO-1 are likely mediated by the end-products of heme degradation, biliverdin / bilirubin and carbon monoxide.

Biliverdin / Bilirubin

Biliverdin and bilirubin exert protective effects by well-known anti-oxidant properties (Stocker et al. 1987). Biliverdin is converted to bilirubin by biliverdin reductase, with the bilirubin being recycled back into biliverdin. This reaction suggests an amplification of the anti-oxidant effects (Stocker et al. 1987).

Biliverdin has been demonstrated to inhibit human complement activation and the proliferation of vascular smooth muscle cells (VSMC) (Nakagami et al, 1993; Ollinger et al, 2005). Kenichiro Yamashita and colleagues have tested biliverdin in allograft transplantation and in *in vitro* T cell proliferation assays. They found that i) biliverdin application (50 µmol/kg/dose) significantly increased cardiac allograft survival whereas the graft infiltration with $CD8^+$ and $CD4^+$ T cells was strongly reduced, ii) the splenocyte proliferation of transplant recipients receiving biliverdin was suppressed, iii) biliverdin inhibited the *in vitro* T cell proliferation stimulated with anti-CD3 and anti-CD28, and iv) biliverdin inhibited the IL-2 secretion of primary T cells stimulated with anti-CD3 and anti-CD28 by suppression of NFkB and NFAT activation (Yamashita et al, 2004).

Bilirubin was considered as a toxic waste product until 1987 because neonatal hyperbilirubinemia could cause bilirubin encephalopathy, also known as “kernicterus”, until its anti-oxidant potential was

recognized (Neuzil et al, 2004). It has been published that individuals with high-normal or supranormal levels of bilirubin in plasma have a lesser incidence of atherosclerosis-related diseases due to its inhibitory effects on injury-induced vascular smooth muscle cell (VSMC) proliferation. Similar results were described for rat and mice VSCM, which are inhibited by bilirubin / biliverdin via cell cycle arrest (Ollinger et al, 2005). Moreover, individuals with above normal bilirubin concentrations in plasma (Gilbert Syndrome) have a lesser incidence of coronary heart disease and carotid plaque formation (Novotny et al, 2003). Reoxygenation of hypoxic cardiomyocytes leads to marked injury which can be strongly reduced by incubation with bilirubin (Foresti et al, 2001).

Immunosuppressive effects of enhanced bilirubin levels have been shown in patients with obstructive jaundice. So, their T cells are characterized by a significantly diminished proliferation (Li et al, 1992). Bilirubin ameliorates post-ischemic myocardial dysfunction (Foresti et al, 2001) and protects the neuronal cells against apoptosis (Dore et al, 1999). Additional, bilirubin inhibits protein kinase C (PKC), the cAMP-dependent protein kinase and the NADPH-dependent oxidase (Kwak et al, 1991; Sano et al, 1985).

An increase in bilirubin concentration has been shown after *in vitro* HO-1 gene transfection of human and murine endothelial cells (Quan et al, 2001). Mice treated with the HO-1 inducer Co-PPIX show enhanced bilirubin levels in serum (Woo et al, 1998). Stimulation of vascular smooth-muscle cells with hemin, a potent inducer of HO-1 gene, increases HO-1 protein expression, HO-1 enzymatic activity (bilirubin), and the observed resistance to oxidative cell injury was manifested only when cells actively produced bilirubin. Tin protoporphyrine IX (Sn-PPIX), an inhibitor of heme oxygenase activity, significantly reduced bilirubin generation and reversed cellular protection afforded by hemin. Moreover, also the exogenous addition of bilirubin to culture medium reduced the cytotoxicity produced by the oxidants (Clark et al, 2000a).

Carbon monoxide

Carbon monoxide (CO) is the gaseous end-product of heme degradation. Two key signaling systems seems to be involved in the response to CO: guanylyl cyclase-cyclic cGMP and p38 mitogen-activated protein kinase (MAPK). CO stimulates the cGMP production by guanylyl cyclase, and cGMP is involved in the inhibition of cell growth (Arias-Diaz et al, 1995), in the regulation of different kinases, phosphodiesterases and ion channels (Degerman et al, 1996; Finn et al, 1996; Lincoln et al, 1995; Otterbein et al, 2003; Palacios et al, 1995; Vaandrager et al, 1996).

CO-mediated increase in cGMP causes systemic vasodilation. Accordingly CO plays an important role in regulation of blood pressure (Sammut et al, 1998). Furthermore, CO shows anti-apoptotic effects in fibroblasts by activation of cGMP (Gerard et al, 1993), and CO has anti-proliferative effects on smooth muscle cells and anti-thrombotic effects in platelets by activation of cGMP and p38 (Petrache et al, 2000; Soares et al, 1999). CO inhibits the secretion of TNF-α, IL-1-β and increases the expression of IL-10 in macrophages through activation of p38 MAPK (especially α and β) and by activation

of guanylyl cyclase with generation of cGMP (Yachie et al, 1999). Treatment of mice with CO led to reduction of TNF-α in serum and induction of IL-10 (Otterbein et al, 2000).
An enhanced release of CO was detected in HO-1-transduced endothelial cells, which show overexpression of HO-1 (Quan et al, 2001).

1.1.2.2 HO-1 increase in pathophysiological conditions and interventions

Hanselmann and colleagues (Hanselmann et al, 2001) observed remarkable HO-1 mRNA and protein inductions in full-thickness excisional wounds in mice. After wound healing the HO-1 expression declined to basal levels. They also showed HO-1 induction in psoriasis. In UVA irradiated mouse skin, Allanson and colleagues saw also a strong HO-1 induction (Allanson et al, 2004).

In rats, heat shock leads to overexpression of HO-1 in liver, spleen and heart and the strongest HO-1 expression is detected in resident macrophages. Heat shock-treated macrophages of mice show an increase of IL-10 secretion. In contrast, the production of the proinflammatory cytokines IL-12p40, IL-6 and TNF-α decreases (Ensor et al, 1994; Raju et al, 1994; Snyder et al, 1992).

HO-1 induction and subsequent antiinflammatory effects directing resolution of inflammation have been observed in animal models for endotoxin shock and sepsis (Poss et al, 1997a; Suzuki et al, 2000; Wiesel et al, 2000), ischemia / reperfusion injury (Akamatsu et al, 2004), experimental autoimmune encephalitis (Maines, 2002), inflammatory bowel diseases (Guo et al, 2001), asthma (Kitada et al, 2001), and rheumatoid arthritis (Hildebrandt et al, 2003). HO-1 induction was also observed in inflammation and acute transplant rejection (Agarwal et al, 1996; Cantoni et al, 1991; Mitani et al, 1992; Willis et al, 1996).

1.1.2.3 The physiological role of HO-1 in inflammation and tissue injury

The physiological increase of HO-1 activity - induced by acute inflammation, chronic diseases, tissue injury, stress in general – is involved in the protective and antiinflammatory response to cellular stress such as ischemia, hypoxia and inflammation. The inhibition of inflammation is likely mediated by the end-products of heme degradation, which e.g. suppress the secretion of proinflammatory mediators and induce antiinflammatory cytokines (see 1.1.2.1). It has been postulated that the physiological HO-1 expression leads to reduction / resolution of inflammation because specific inhibition of HO-1 expression results in increased inflammation.

This physiological significance of HO-1 has been proven in several experiments, by which the inhibition of HO-1 impairs the experimental outcome of diseases. For example, hearts transplanted to immunosuppressed recipients are rapidly rejected by application of the HO-1 specific inhibitor Sn-PPIX

or by HO-1 deficiency (Otterbein et al, 2003). In a rat model for experimental autoimmune encephalomyelitis (EAE) high expression of HO-1 has been detected in cerebral lesions. Application of hemin, an inducer of HO-1, inhibited EAE development effectively and Sn-PPIX, an inhibitor of HO-1, markedly exacerbated EAE (Liu et al, 2001).

The physiological importance of HO-1 has been confirmed in HO-1 knockout mice (see 1.1.2.4) and in a patient with HO-1 deficiency. HO-1 deficiency both, in mice and human, results in similar abnormal phenotypes with a progressive chronic inflammation and a reduced cellular resistance to oxidative stress (Poss et al, 1997b; Yachie et al, 1999).

These data indicate that endogenous HO-1 activity plays an important protective role. The antiinflammatory efficacy of HO-1 seems to be dependent on the severity of the disease. So, the physiologic induction of HO-1 may sufficient for e.g. skin repair but may be to weak or to late in exacerbated inflammation (e.g. in autoimmune diseases). Therefore a targeted superinduction of HO-1 e.g. by pharmacologic intervention may represent a promising approach for the treatment of inflammatory disorders.

1.1.2.4 HO-1 gene transfer, knockout and knockdown

Several studies have shown that HO-1 has antiinflammatory and protective properties (Nath et al, 1992; Willis et al, 1996) and that HO-1 deficiency results in chronic inflammation (Poss et al, 1997b; Yachie et al, 1999). Accordingly HO-1 gene transfer and knockdown / knockout have been used to prevent inflammatory injury or to establish the antiinflammatory role of endogenous HO-1.

Transfection of the heart endothelium with HO-1 gene leads to detoxification of the effects of heme and heme protein (Abraham et al, 1995). HO-1-transgenic mice show neuroprotection in stroke model. Moreover, HO-1 gene transfer mediates protection against tissue injury in myocardial infarction, liver ischemia / reperfusion, in hyperoxygen- and endotoxin-induced lung injury (Amersi et al, 1999; Inoue et al, 2001; Melo et al, 2002; Otterbein et al, 1999; Panahian et al, 1999). Mouse macrophages transduced with HO-1 vector show reduced TNF-α secretion. Additionally increased levels of bilirubin and CO were found in supernatants (Otterbein et al, 2000). Intranasal lung-specific siRNA administration against HO-1 leads to enhanced apoptosis via increased Fas expression and caspase 3 activity in mouse lung during ischemia/reperfusion-induced lung injury. Apoptosis was also induced *in vitro* in siRNA treated endothelial cells. Attenuated apoptosis was detected after HO-1 overexpression (Zhang et al, 2004).

As already mentioned, the physiological role of HO-1 was convinced in HO-1 knockout mice. These mice are characterized by deposition of iron in liver and spleen. They show tissue injury, chronic in-

flammation and reduced cellular resistance against oxidative and inflammatory stress. Treatment with LPS results in strong increase in liver necrosis, anemia and high mortality in contrast to wild type mice. Cultured Fibroblasts of these mice are very sensitive to the toxic effects of heme. This clinical picture is similar to the HO-1 human deficiency, which showed anemia, iron deposition and chronic inflammation of kidney and liver (Poss et al, 1997a; Poss et al, 1997b; Wiesel et al, 2000).

1.1.2.5 Inducers and inhibitors of HO-1

The substrate of HO-1, Fe protoporphyrin IX (Fe-PPIX, heme, hemin) is a multifunctional molecule in nature. The porphyrin ring is a tetra dentate ligand that forms a complex with several metals such as Fe, Cu, Zn, Sn and Co (Smith et al, 1975). The binding pocket of HO-1 has specificity toward the side chains of the porphyrin ring and does not recognize the metal moiety of the molecule. Metalloporphyrins, in which the heme-iron has been replaced by other metals, like Zn, Sn, and Co, can compete for heme and inhibit the activity of the enzyme because they cannot be degraded to bile pigments (Maines, 1981). Cobalt (III) protoporphyrin IX (Co-PPIX) is a strong HO-1 inducer used in different experimental settings (Rosenberg, 1993; Sardana et al, 1987; Shan et al, 2000).

In contrast to Co-PPIX, Sn-PPIX and Zn-PPIX are only weak inducers of HO-1 expression but markedly decrease its activity, which explains their usage as potent HO-1 inhibitors (Sardana et al, 1987). The molecules hemin (Fe-PPIX) and Co-PPIX are well-known HO-1 inducers which display protective effects in inflammation, autoimmune and in transplantation models (Amersi et al, 1999; Clark et al, 2000b; DeBruyne et al, 2000; Liu et al, 2001; Woo et al, 2000).

Effects of HO-1 inducers

Acute inflammatory responses and tissue injury are suppressed by treatment with HO-1 inducers (heme, metalloporphyrins, heavy metals). This suppression correlates with inhibition of E- and P-selectin expression in spleen after *in vivo* stimulation. In contrast, administration of Zn-PPIX upregulates the adhesion molecule expression (Vachharajani et al, 2000).
Hemin inhibits the EAE (experimental autoimmune encephalomyelitis) model in mice with reduction of central injury (Liu et al, 2001). Treatment of bovine vascular smooth muscle cells with hemin leads to enhanced HO-1 expression and bilirubin in supernatants (Clark et al, 2000b).
The capacity of Co-PPIX to induce HO-1 *in vivo* has been described for spleen, liver and kidney of treated mice. Co-PPIX leads to reduction of T cells in spleen and in *ex vivo* tests to reduction of T cell proliferation in MLR without suppression of IL-2 secretion. The decrease of T cell and NK cell cytotoxicity by Co-PPIX was abolished by Zn-PPIX, an inhibitor of HO-1 (Woo et al, 1998). Co-PPIX

treatment results in survival of heart transplants in mice and shows a higher potential for HO-1 induction than hemin (Shan et al, 2000; Woo et al, 1998).
Curcumin is an antiinflammatory active molecule from *Curcuma long inn.* which is a potent HO-1 inducer and has shown therapeutic effects in psoriasis (Chang, 2001; Heng et al, 2000; Scapagnini et al, 2002). Fumaric acid esters, the most prescribed drug for psoriasis treatment in Germany, is also a strong HO-1 inducer with antiinflammatory properties (Lehmann et al, in Review).

Effects of HO-1 inhibitors

Mice treated with HO-1 inhibitor (Sn-PPIX) suffer from inflammatory reaction, tissue injury and show increased acute transplant rejection (Clark et al, 2000b; Liu et al, 2001; Sato et al, 2001; Shimizu et al, 2000).
Sn-PPIX has been shown to reduce significantly the bilirubin generation and to reverse the protection of VSMC against oxidative stress afforded by hemin (Clark et al, 2000a). Interesting findings have been also made with heart transplants: Mouse hearts transplanted to immunosuppressed (cobra venom factor, cyclosporine A) rats survive indefinitely only if HO-1 activity is not suppressed by Sn-PPIX or not defect. This indicates the necessity of HO-1 for prevention of heart rejection (Koyamada et al, 1998; Sato et al, 2001; Soares et al, 1998; Soares et al, 2001).

1.1.2.6 HO-1 induction in antigen-presenting cells

HO-1 induction is considered as an important counter-regulatory mechanism for the limitation of inflammation and overwhelming immune activation. An important role of monocytes/macrophages in this antiinflammatory and immunosuppressive activity is indicated by reports showing HO-1 induction in alveolar macrophages of mice in allergic airway inflammation (Kitada et al, 2001), in macrophages and circulating monocytes from patients after coronary artery bypass graft surgery (Philippidis et al, 2004), and in murine macrophages after IL-10 treatment (Lee et al, 2002).
Kampfer and colleagues observed a rapid (6 hours) and strong increase of HO-1 at the wound site of skin injured mice, in diabetes mice the HO-1 elevation was later. Their immunohistochemistry revealed infiltrating monocytic cells as the predominant and major source of HO-1 at the wound site. They reported similar HO-1 induction by nitric oxide in RAW 264.7 macrophages (Kampfer et al, 2001).

1.1.3 Principle of HO-1 induction as therapeutic or prophylactic anti-inflammatory approach

The numerous results for the antiinflammatory and cytoprotective function of HO-1 induction in various *in vivo* and *in vitro* assays lead to the conclusion, that HO-1 overexpression (transduction or induction by pharmacological molecules) may play a pivotal role in approaches against inflammation and transplant rejection. HO-1 induction confers a high physiological protection against inflammatory injury and acts by two mechanisms:

i. HO-1 induction in immune cells (e.g. antigen-presenting cells) inhibits the induction of inflammation and specific immune reaction to a stimulus, and
ii. HO-1 induction in target cells (e.g. endothelium) protects them against released inflammatory mediators (e.g. protection from apoptosis, cellular activation).

This therapeutic principle of HO-1 induction has been successfully validated in *in vivo* and *in vitro* tests (see above).

The synthetic and natural molecules for HO-1 induction as well as transduction have - contrary to the natural stressful inducers – the advantage of HO-1 induction without additional cellular stress and tissue damage. Consequently the HO-1 induction is achieved primarily and not secondarily as result of stress.
Figure 2 shows an immunotherapy (right scheme) with HO-1 superinduction in contrast to the physiological HO-1 expression by cellular stress conditions (left scheme). With “stress-free” drugs a therapy for modulation of immune and inflammatory system is imaginable, by which the HO-1 is up-regulated without stress and tissue injury and mediates inhibition of inflammation as well as cell protection. The indications for this therapy could be persistent pathophysiological conditions (autoimmune diseases) as well as prophylactic therapy (transplantations, surgery). The physiological HO-1 expression (e.g. Psoriasis, EAE) induced by cellular stress / disease conditions seems to be to weak to resolve the inflammatory conditions.

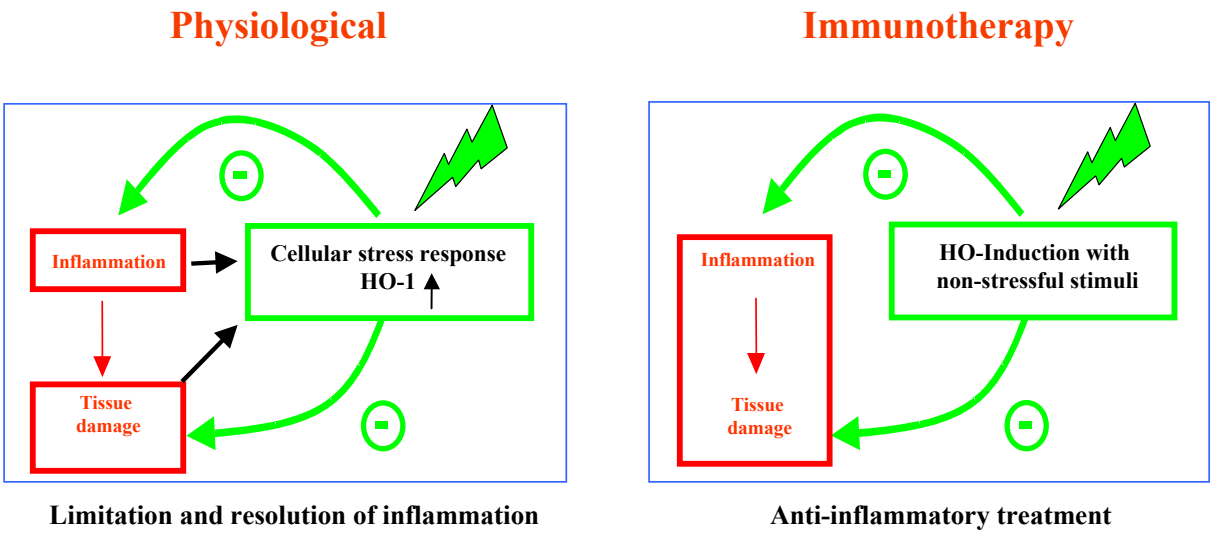


Figure 2. The physiological role of HO-1 and HO-1 induction as immune therapy.
The physiological function of HO-1 consists in limitation and resolution of inflammation after HO-1 induction with cellular stress e.g. inflammation, tissue damage. In contrast, the HO-1 immunotherapy bases on HO-1 induction without stress and the limitation and resolution of persistent diseases or antiinflammatory treatment as prophylaxis.

1.2 Immune system and regulation

1.2.1 The immune system

The immune system of mammalia consists of the innate and adaptive immune responses.
Innate immunity refers to nonspecific defense mechanisms that come into play immediately or within hours of an pathogen appearance in the body. These mechanisms include physical barriers such as skin, effector molecules in body fluids such as mucosal secretions and blood, and immune cells of the myeloid and NK cell lineages (Janeway et al, 2002). The innate immune response is activated by chemical properties of molecules / conserved pathogen-associated molecular patterns e.g. by the danger signals via Toll-like receptors (TLR), which lead via cytokine induction in antigen presenting cells to activation of the adaptive immunity. The evolutionarily conserved sentinel TLRs belong to the TLR / IL-1 receptor (TIR) family, and recognize bacterial/viral-specific pathogen-associated molecular patterns (Nedzhitov et al, 1997; Johnson et al, 2003).
TLRs trigger host inflammatory responses that are mediated by monocytes, macrophages, dendritic cells, neutrophils and granulocytes. High TLR expression has been demonstrated in airway epithelium and skin which represent important sites of host / pathogen interaction (McInturff et al, 2005). The induced cytokine mediators by TLRs may then activate systemic responses to recruit leukocytes to the site of inflammation. Known TLRs are: TLR2 in recognition of microbial lipopeptides, TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, TLR3 for viral double-stranded RNA, TLR5 for flagellin, TLR9 for CpG DNA, TLR4 for LPS and TLR10 with unknown functional significance (Hasan et al, 2005; Takeda et al, 2004).
Adaptive immunity refers to antigen-specific immune responses. The adaptive immune response is more complex than the innate. The antigen first must be processed and presented. Once an antigen has been recognized, the adaptive immune system expands and maturates a set of immune cells and antibodies specifically designed to attack that antigen or the antigen bearing pathogen within days or weeks. The adaptive immunity includes a memory that renders future responses against a specific antigen more efficient. Innate and adaptive immunity are closely inter-connected. Mononuclear phagocytes (monocytes, macrophages, dendritic cells) are cells of the innate immune response, which uptake and disturb the pathogen and regulate the immunoreaction of other cells with secretion of e.g. cytokines and with expression of different receptors and molecules. Moreover, they are involved in the adaptive, specific immune response by the presentation of the antigen within MHCII / MHCI toward T-helper ($CD4^+$) and cytotoxic $CD8^+$ T cells (Janeway et al, 2002).

$CD4^+$ and $CD8^+$ T cells as well as B cells and antibodies belong to the specific, adaptive immunity. The activation of $CD4^+$ T-helper cells occurs by the presence of two signals: the recognition of the MHCII-presented antigens and the costimulation with e.g. CD86 on the antigen-presenting cell (APC). The presence of two signals leads to the successful activation of T cells, which than differentiate and proliferate to effector cells. The activated T lymphocytes can than secrete cytokines and exert cytotoxic activity. The cytotoxic T cells are especially the $CD8^+$ T cells, which recognize the intracellular antigen within MHCI molecule – a molecule which is expressed on all nuclear cells (Janeway et al, 2002).

The unspecific, innate and the specific, adaptive cellular immune functions are regulated by cytokines and by interaction of membrane molecules. Dysbalances in the immune regulation can lead to pathophysiological states and are targets for experimental and clinical immune therapy.

1.2.1.1 Regulation of innate immunity

An inflammation develops e.g. by reaction of the body against the penetration of an infectious agent, by ischemia/hypoxia, tissue traumata, by physical injury. An inflammation is characterized by increase of blood supply into the injured tissue, enhancement of capillary permeability, retraction of endothelial cells and emigration of leukocytes from endothelial capillaries into the surrounding tissue. Due to the increase of the capillary permeability the antibodies, complement and molecules of plasma system can advance into the inflammation focus. An inflammation is steered by plasma enzymes, vasoactive mediators and by cytokines (Janeway et al, 2002).

1.2.1.1.1 Cytokine regulation of the inflammatory response

Cytokines play an essential role for spreading and amplification of the inflammatory response. On the beginning of inflammation ("first wave") cytokines such as TNF-α, IL-1β and the chemokine IL-8 can be secreted by resident macrophages, activated parenchyma cells and stroma cells of the tissue. After activation of endothelial cells and secretion of chemoattractant molecules, granulocytes and mononuclear cells can infiltrate into the inflammation site and secrete cytokines ("second wave"), which recruit further leukocytes to the inflamed tissue. The inflammation is amplified and can result in severe tissue injury. The inflammation is therefore limited and resolved by antiinflammatory cytokines. One example is the kinetics of pro- and antiinflammatory cytokines after endotoxin stimulation of monocytes / macrophages. Initially proinflammatory cytokines such as TNF-α, IL-1β and IL-12 are produced. TNF-α increases the secretion of further proinflammatory cytokines, e.g. IL-6 and IL-8. IL-12 enhances the production of the Th1 cytokine IFN-γ, which heighten again IL-12 and TNF-α and acts synergistically with TNF-α.

This proinflammatory wave is followed by the monocytic secretion of antiinflammatory cytokines. IL-10 inhibits the production of TNF-α, IL-12 and also of IFN-γ. The IL-1 receptor-antagonist interferes with IL-1 action by competition at the receptor. Soluble TNF-α receptors neutralize the function of TNF-α. IL-10 inhibits the secretion of the proinflammatory cytokines and also of itself and therefore participates in limitation of the pro- and antiinflammatory stage of the inflammation reaction (Janeway et al, 2002).
In addition to the autoregulation of inflammation further limitation systems exist. For example, switch off of inflammatory response by phagocytosis of apoptotic cells, activation-induced death of inflammatory cells, and production of antiinflammatory hormones (glucocorticoids, catecholamines) after activation of the neuroendocrine stress response by TNF-α and IL-1β. These hormones can suppress the production of inflammatory cytokines and increase the secretion of IL-10 (Janeway et al, 2002).

So, the innate system is essentially regulated by the balance of pro- and antiinflammatory cytokines. For example activated monocytes / macrophages secrete proinflammatory mediators such as TNF-α, IL-1β, IL-12 and IL-18 as well as the antiinflammatory cytokines IL-10 and IL-1RA. The balance between proinflammatory and antiinflammatory mediators crucially defines the status of the immune system. Therefore the overexpression of proinflammatory mediators (e.g. TNF-α) may lead to systemic inflammation with adverse consequences. In opposite, the prominent expression of the antiinflammatory and immune suppressive cytokine IL-10 directs to hypo-inflammation and immune depression.

1.2.1.2 Regulation of adaptive immunity

The adaptive immune system consists of the cellular and antibody mediated immunity. Both branches of immunity are regulated antagonistically during T cell differentiation by cytokines.

1.2.1.2.1 Th1 / Th2 lymphocyte regulation

Naive T-helper cells develop after antigen stimulation in presence of IL-12 into Th1 cells. The signature cytokines of Th1 cells are IL-2, TNF-α and IFN-γ. Th1 cells are the most important T cells for stimulation of phagocytosis-mediated immunity, the delayed-type hypersensitivity, and for anti-tumor and anti-virus defense. The type 1 cytokine pattern is proinflammatory, anti-viral, anti-tumor and plays a great role in cell mediated acute transplant rejection and in abortion. Th1 cell-triggered Ig-production (IgG1 and IgG3 in human) activates complement and improves phagocytosis. Th1 cytokines are detected in excess in many autoimmune diseases (Morbus Crohn, rheumatoid arthritis, psoriasis) (Ardizzone et al, 2005; Radeke et al, 2005; Toubi et al, 2005).

Development of Th2 cells from naive T-helper cells occurs by antigen presentation (allergen) in the presence of IL-4. Th2 cytokine pattern consists of IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 and is responsible for the antibody-mediated immune response. Th2 cytokines induces IgE production (IL-4), IgA synthesis and the activation of eosinophils (IL-5) and mast cells. Th2 cells support B cells for proliferation, differentiation and antibody production. Diseases, which are characterized by Th2 cells, are lupus erythematodes, allergic and atopic diseases (Chen et al, 2005; Maizels, 2005; Mouzaki et al, 2005; Radeke et al, 2005).

Conditions, which define the differentiation to Th1 or Th2 cells are: cytokine pattern, the activation status of the APCs and the concentration and status of antigen. The predominance of IL12 / IFN-γ results in Th1 differentiation and IL-4 / IL-10 support Th2 differentiation. Both cytokine groups work antagonistically. So, IL-4 enhances the IgE and IgG1 production in mice, and IFN-γ suppresses both (Ouyang et al, 1998; Seder et al, 1994; Szabo et al, 1997). IFN-gamma promotes Th1 development by inducing IL-12 production from activated macrophages and IL-12 receptor expression on antigen-activated T helper cells, while directly inhibiting the growth of Th2 cells (Gajewski et al, 1989; Ma et al, 1996; Szabo et al, 1997). IL-10, a cytokine which is preferentially produced by Th2 cells (and regulatory T cells), inhibits IL-12 production by activated macrophages, thus indirectly inhibiting Th1 development (Fiorentino et al, 1991).

1.2.1.2.2 Dendritic cells

Dendritic cells are classified into follicular and interdigitating dendritic cells (DC). Follicular DC play an important role in controlling of B cell maturation, isotype switching and memory. Until today their ontogeny has remained unresolved (Heinemann et al, 2005). The interdigitating DC are subclassified in myeloid and plasmacytoid DC (Banchereau et al, 2003; Howard CJ et al, 2004; Ito et al, 2005). Plasmacytoid DC are CD11c-negative, produce type I interferons in response to viruses and play a critical role in antiviral immunity but also in inflammation (Banchereau et al, 2003; Ito et al, 2005). The myeloid DC include Langerhans cells and interstitial DC, which are positive for CD11c and produce IL-12. In contrast to Langerhans cells the interstitial DC produce IL-10 and induce naïve B cell differentiation (Banchereau et al, 2003).

Classical antigen-presenting cells (APC) like myeloid DC induce activation of T cells. The DC residue in an immature state in non-lymphoid tissue (e.g. Langerhans cells in epidermis, interstitial DC in dermis) where they efficiently capture foreign antigens (Ag). Upon activation by pathogens or by inflammatory stimuli like TNF-alpha or IL-1ß, DC migrate to lymphoid organs where they potently activate Ag-specific T cell responses (Banchereau et al, 1998; Kalinski et al, 1999).

During migration, DC undergo functional and phenotypic changes termed as “maturation”. They up-regulate surface molecules like MHC class I and II molecules, CD86, CD40, and CD54, and thus become potent inducers of T cell activation. Maturation is further driven by CD40-CD40 ligand

interaction upon contact of DC with T cells (Cella et al, 1996). In parallel, DC lose their capacity for endocytosis and *de novo* MHC class II synthesis during migration to the lymphoid tissues (Kampgen et al, 1991; Sallusto et al, 1995).

This feature enables mature DC to preserve the Ag taken-up in the periphery for Ag-specific T cell activation. In addition, mature DC are able to release high amounts of proinflammatory cytokines like TNF-α or IL-1ß and of the Th1 promoting cytokines IL-12 and IL-18 but they also secrete the counter-regulatory cytokine IL-10 (Cella et al, 1996; De Saint-Vis et al, 1998; Stoll et al, 1998; Trinchieri, 1995). Therefore, DC are not only unique in induction of naive T cell activation but also play a decisive role in T-helper cell polarization.

1.2.1.3 The counterbalance of innate versus adaptive immunity on APC level

One basis for the regulation of innate versus adaptive immunity is the differentiation status of APC. Monocytes can differentiate into macrophages, which are important scavenger cells, or to myeloid dendritic cells, which are the most effective antigen-presenting cells for the promotion / initiation of the adaptive immune response. The monocytic differentiation to either macrophages or dendritic cells is characterized by expression of different antigens and is regulated antagonistically similar to Th1 and Th2 T-helper cell differentiation. So, regulatory mediators can enhance macrophage differentiation and inhibit DC maturation or conversely. States with suppressed dendritic cell maturation (e.g. exposure to IL-10 and apoptotic cells) promote the macrophage and immature DC status and exert immune depression.

Common for all regulation types of the immune system is that dysbalance leads to pathophysiological diseases / injury and can be target for therapeutic intervention (Ardizzone et al, 2005; Bos et al, 2005; Brok et al, 2002; Chen et al, 2005; Den Broeder et al, 2002; Feldmann, 2002; Kaufman et al, 2004; Maizels, 2005; Mouzaki et al, 2005; Ogilvie et al, 2001; Radeke et al, 2005; Rosmarin et al, 2005; Schon, 1999; Schon et al, 2005; Schottelius et al, 2004; Toubi et al, 2005).

1.2.2 Cytokines

Cytokines are proteins which mediate the interaction between cells. They are secreted after adequate stimulation and act via their respective receptors (Abbas et al, 1996). The action of cytokines is pleiotropic and redundant, and their effects can be autocrine, paracrine and endocrine. Cytokines are crucially involved in regulation of immunity and inflammation. There are proinflammatory cytokines (e.g. TNF-α, IL-1-β), antiinflammatory cytokines (e.g. IL-10, IL-1RA), promoters of cellular immunity (e.g. the type 1 cytokines IFN-γ and IL-12), mediators of antibody-mediated immune response (e.g. the type 2 cytokines IL-4 and IL-5), and regulators of hematopoiesis (e.g. IL-3, M-CSF, GM-CSF). A strict demarcation of the cytokine functions is difficult because of overlapping and recip-

rocal interaction. The quantity of the secreted cytokines and the cytokine profile are indicators of the competence and the regulation of the immune system (Janeway et al, 2002).

1.2.2.1 TNF-α

TNF-α is the most important inflammatory cytokine. It is a 51 kDa weighty homotrimer, whose action is mediated via two receptors (55 and 75 kDa). TNF-α is produced by non-specific stimulated mononuclear phagocytes, NK cells, mast cells, and Ag-stimulated T cells. The effects of TNF-α are: increase of the expression of adhesion molecules on endothelial cells, enhancement of phagocytosis, upregulation of cytokine production and release, induction of acute phase proteins, stress response and fever, and participation in cellular defense against viruses. TNF-α is responsible for the induction of septic shock, which is characterized by decrease of blood pressure, injury of endothelium and promotion of microthrombosis (Janeway et al, 2002).
In summary, TNF-α shows strong proinflammatory properties and participates essentially to tissue damage in autoimmune diseases and innate inflammation. Its key role is underscored by the therapeutic efficacy of molecules that interfere with TNF-α functions (Schon et al, 2005; Schottelius et al, 2004).

1.2.2.2 IL-10

The antiinflammatory cytokine IL-10 is a 18 kDa, which also inhibits cellular immunity. It decreases the cytokine production, the MHCII expression and the accessory functions of APC. IL-10 is predominantly expressed by monocytes / macrophages but also by lymphocytes (B cells, Th2 cells, regulatory T cells) and stimulated keratinocytes. The release of IL-10 by phagocytes can be induced by proinflammatory cytokines (TNF-α) and TLR ligands (e.g. LPS). IL-10 shows negative regulation and limitation of inflammatory reaction by inhibition of TNF-α and IL-12 secretion of monocytes.
IL-10 suppresses the development of monocytes into dendritic cells and promotes macrophage differentiation. It upregulates the production/secretion of the Type-2 cytokine IL-4 and down-regulates the type 1 cytokines, IFN-γ and IL-12, thereby supporting the differentiation of Th2 cells and inhibiting Th1 differentiation (Janeway et al, 2002; Jung et al, 2004).

1.2.2.3 IL-12

IL-12 is a heterodimeric cytokine (35 and 40 kDa), which is released by activated (LPS, lipoproteins, IFN-γ) monocytes, macrophages, B cells and dendritic cells. Its receptors are IL-12Rß1 (p40 chain) and ß2 (p35 chain). IL-12 activates the cytolytic activity and IFN-γ production of T cells and NK cells and induces the differentiation of naive T cells into IFN-γ and IL-2 producing Th1 cells. It is a positive

regulator for cellular responses and for inflammation by increasing the production of the monocyte activator IFN-γ (Janeway et al, 2002).

Recently new members of IL-12 family have been discovered: IL-23 (p40, p19) and IL-27 (p28, EBI3) (Hunter, 2005). IL-23 has been identified as a strong IL-17 inducer in T cells and plays a dominant role in autoimmune diseases. So, IL-23 may be an even better candidate for therapeutic interventions than IL-12 (Gran et al, 2004; Rosmarin et al, 2005).

1.2.2.4 IL-18

Interleukin 18 (IL-18), a member of the IL-1 cytokine family, is a 18 kDa proinflammatory cytokine which has been identified as a costimulatory factor for production of IFN-γ. It is synthesized as a 24 kDa precursor molecule without a signaling peptide and must be cleaved by IL-1β converting enzyme (Caspase-1) at aspartic acid to produce a mature, bioactive molecule that is readily released from activated monocytes, macrophages, dendritic cells, keratinocytes, intestinal epithelial cells and Kupffer cells (Gu et al, 1997; Okamura et al, 1995).
IL-18 shares functional similarities with IL-12. IL-18 acts on T helper cells (Th1) and in combination with IL-12 strongly induces production of IFN-γ by activated T and NK cells which plays a critical role in macrophage activation, B cell maturation and induction of MHC class I and II antigen expression. Furthermore, IL-18 enhances IL-2, GM-CSF production and Fas ligand expression in Th1 cells. Two IL-18 receptors have been identified (IL-18Rα, IL-18Rβ) and both are important for IL-18 signaling (Gu et al, 1997; Okamura et al, 1995).
IL-18 has been reported to play an important role in the pathophysiology of psoriasis (Ohta et al, 2001), rheumatoid arthritis (Bresnihan et al, 2002), experimental autoimmune encephalomyelitis (EAE) (Ito et al, 2003), atherosclerosis (Mallat et al, 2001), and systemic lupus erythematosus (Wong et al, 2002). Interestingly, the potent antiinflammatory cytokine IL-10 fails to inhibit IL-18 production in response to LPS and IFNγ in macrophages (Zediak et al, 2003).

1.2.2.5 IL-2

IL-2 is a 14-17 kDa T cell cytokine, which is induced after Ag stimulation especially in naive and Th1 cells. The high-affine IL-2 receptor consists of three chains (CD25, CD122, CD132) and is expressed on activated T cells and other cells like monocytes. IL-2 is an autocrine and paracrine growth factor, which directs the T cells from the G1 into the S phase of the mitosis and therefore is essential for T cell proliferation after mitogenic stimulation. Enhanced IL-2 production / secretion correlates with different inflammatory conditions (Ortonne, 1999).

1.2.2.6 IFN-γ

IFN-γ is a homodimer, consisting of 21 and 24 kDa chains. It is produced by Th1 and by NK cells. The expression of IFN-γ after Ag-stimulation is increased by IL-2, IL-12 and IL-18. Additionally, IL-23, the new cytokine of the IL-12 family, enhances the secretion of IFN-γ.
IFN-γ stimulates the differentiation of naive Th0 cells into Th1 cells and inhibits the development of Th2 cells. It is a strong activator of monocytes by enhancing their MHCII expression, Ag presentation, production of proinflammatory cytokines and defense against intracellular pathogens. IFN-γ induces an anti-viral and anti-proliferative state of the cells. Taken together IFN-γ is an important cytokine for the cellular immune response and also for inflammation. Its role in type 1-autoimmunity is predominant (Janeway et al, 2002; Schon et al, 2005).

1.2.2.7 IL-4

The most important physiological role of the type 2 cytokine IL-4 is the regulation of antibody-mediated immunity. IL-4 is produced by Th2 T cells, by activated mast cells and basophilic granulocytes. It is required for the promotion of IgE synthesis and therefore for the defense against parasites (e.g. helminthes). It is mediator of the type 1 hypersensitivity reactions and a growth and differentiation factor of activated Th2 cells. It plays a critical role in the counterbalance of Th1 and Th2 T-helper cell differentiation (Chen et al, 2005; Janeway et al, 2002; Maizels, 2005; Mouzaki et al, 2005; Radeke et al, 2005).

1.2.3 T cell proliferation

T cell proliferation is a response of adaptive immunity to allogeneic MHC antigens and to autologous MHC with degraded foreign or host Ag (autoimmune diseases). T cell proliferation is an indicator of immune reactivity and can be used for diagnostics in allotransplantation and vaccination and for drug discovery.

1.2.3.1 Mixed leukocyte reaction (MLR)

MLR is an *in vitro* method which originates from the transplant diagnostics for determination of recipient transplant compatibility. The method bases on T cell stimulation – without previous immunization – of two histocompatible individuals. The important antigen-presenting cells in MLR are dendritic cells. In MLR, T cells are directly activated by allogeneic MHC molecules. Dependent on the differences in the MHCI or MHCII of the allogeneic individuals there arises a predominance of either $CD4^+$ or $CD8^+$ T cells. IL-2, IFN-γ and TNF-β are the most important cytokines in MLR and essential for the transplant rejection. IL-2 initiates the activation and proliferation of T and B cells.

IFN-γ synergistically with TNF-β activates the antigen-presenting cells. Additionally, *in vivo* IFN-γ and TNF-β increase the expression of MHCI and MHCII on endothelial and parenchyma cells and of adhesion molecules.
In transplant diagnostics a weak recipient anti-donor MLR reaction is associated with a better transplant tolerance. MLR is very important for assessing the risk of "graft versus host disease" in bone marrow transplantation. Also, the application of MLR is qualified for drug tests on alloreactive T cell potency and on the function of antigen-presenting cells. MLR serves as a general *in vitro* model for cellular immunity (Baan et al, 2005; Janeway et al, 2002; Zang et al, 2005).

1.2.3.2 Lymphocyte transformation test (LTT)

Similar to MLR, LTT is an *in vitro* model for cellular immunity, but in opposition to MLR LTT bases on stimulation of memory T cells in response to recall antigens (e.g. tetanus toxoid) presented by autologous antigen-presenting cells. Determination of lymphocyte proliferation in LTT is qualified for assessing an immunization response or for investigation of drug effects on T cell or APC function (Merk, 2005).

1.2.4 Surface receptors on mononuclear phagocytes and dendritic cells

Distinct cell populations and tissues are characterized by the expression pattern of cell surface molecules which changes dependent on activation and differentiation status. Therefore the immunophenotype of cells represents an important parameter for characterization of cell function and can be used for determination of drug effects.
In the following, membrane receptors, whose expression was investigated in this work, are described.

1.2.4.1 CD14

CD14 is a "pattern recognition" receptor on monocytes and macrophages, which binds the complex of LPS-binding protein (LBP) with LPS and presents it to the Toll-like receptor (TLR) 4 mediating signal transduction and secretion of proinflammatory cytokines (Wright et al, 1989; Wright et al, 1990). CD14 is a glycoprotein, which exists both in membrane bound (via glycophosphatidylinositol - GPI) and soluble forms. Cells without membrane CD14 (e.g. endothelial cells) are LPS activated by the soluble CD14 (Pugin et al, 1993).
Phagocytosis by polymorphonuclear granulocytes and monocytes constitutes an essential arm of host defense against bacterial or fungal infections. CD14 mediates the recognition and phagocytosis of apoptotic cells (Devitt et al, 1998) and Monocytes can phagocytose gram-negative bacteria via CD14 receptor (Grunwald et al, 1996).

1.2.4.2 HLA-DR

HLA-DR belongs to the human MHC class II molecules. In human exists three types of MHC class II molecules: HLA-DR, HLA-DP and HLA-DQ. In mice are two types (H-2A, H-2E) published (Janeway et al, 2002).
HLA-DR is constitutively expressed on classical antigen-presenting cells (dendritic cells, monocytes / macrophages, B cells) and also on activated T cells. HLA-DR presents processed extracellular antigens to T-helper cells, and the quantity of HLA-DR molecules is crucial for sufficient initiation of a specific immune reaction (Alberts et al, 2002; Janeway et al, 2002). Reduction or loss of HLA-DR expression are indicators for functional deactivation of APC and are associated with decreased antigen presentation and cytokine secretion (Gruner et al, 1986). HLA-DR expression is antagonistically regulated by cytokines: increase of HLA-DR expression and therefore of cellular immunity by IFN-γ and inhibition by IL-10 (Janeway et al, 2002).

1.2.4.3 CD86

CD86 belongs to the B7-family and is expressed by professional antigen-presenting cells. It is a costimulatory molecule, whose recognition occurs by CD28 on activated T cells and represents the second activation signals for T cells. The loss or inhibition of the CD28 interaction lead to anergy of T cells. CD86 plays a great role in activation and expansion of autoreactive T cells and by infiltration of respiratory tract by eosinophils (Bagenstose et al, 2002; Shi et al, 1999).

1.2.4.4 CD1a

The non-polymorphic CD1 proteins present lipid antigens to T cells. These evolutionary conserved antigen-presenting molecules are divided into group I (CD1a - D1e) and group II (CD1d). CD1 molecules are 43 to 49 kDa cell-surface glycoproteins homologous to MHCI molecules with a limited allelic polymorphism and compared to MHCI with a deeper and more hydrophobic antigen binding groove. Human CD1a, CD1b and CD1c present mammalian and mycobacterial lipids to CD4 and CD8 T cells. CD1d-restricted T cells appear to be primarily self-reactive, and they have been implicated in the control of autoimmune diseases (Han et al, 1999; Joyce et al, 2003; Matsuda et al, 2001; Park et al, 2001; Porcelli et al, 1999; Schaible et al, 2000; Wang et al, 2001).
CD1a is a surface receptor on dendritic cells. Presentation of the antigen (intracellular and extracellular pathogens) occurs by connection of the alkyl-group of the antigen to the hydrophobic groove of CD1a. The hydrophilic sites of the antigen are featured for the T cell contact (Ulrichs et al, 2000).

1.2.4.5 CD83

Human CD83 is a 45-kDa glycoprotein and member of the Ig superfamily localized on cell surface (Kozlow et al, 1993; Zhou et al, 1992). It is an adhesion molecule with a dominant expression on immune competent, activated and mature dendritic cells. CD83 is required for CD4+ T cell generation, because CD83-/- mice had a block in $CD4^+$ positive thymocyte development (Fujimoto et al, 2002).
The molecule is involved in intralesional activation of T cells in psoriasis, and loss or reduction of CD83 is associated with immune depression (Koga et al, 2002).
A soluble CD83 isoform has been described, which is released by activated DCs and B cells and is detectable in normal human sera (Hock et al, 2001). Soluble CD83 inhibits the maturation of dendritic cells (Lechmann et al, 2001).

1.2.5 Immune regulation via differentiation and maturation of antigen-presenting cells

An important group under the antigen-presenting cells are the long-living mononuclear phagocytes / myeloid dendritic cells (see 1.2.1.2.2). They can, dependent on differentiation stage, function as i) dendritic cells with professional and high competent antigen presentation and as ii) macrophages with capable inflammatory activity, phagocytosis and killing of pathogens. For their strong scavenging function (macrophages) and unique capability for competent presentation of antigens to T cells (dendritic cells) they play a key role in inflammation and adaptive immune response, respectively, and for this defensive purpose they are positioned in strategic important places in tissue. Blood monocytes can migrate into the tissue and differentiate either into either macrophages or myeloid dendritic cells (Janeway et al, 2002).

Important receptors on macrophages / monocytes are: LPS / LBP receptor CD14, Fc-gamma receptors CD64, CD32 and CD16 and complement receptors. These receptors participate in regulation of inflammation, in uptake of opsonized and unopsonized pathogens and of apoptotic cells. Monocytes / macrophages are rather incapable antigen-presenting cells, which show only moderate expression of MHCII (e.g. HLA-DR) and costimulatory molecules (e.g. CD86).

Myeloid dendritic cells are accessory cells with high importance in initiation of the primary specific immune reaction. The majority of blood / tissue DC are in an immature state in which they express a repertoire of receptors for efficient antigen uptake (CD11b, CD32, CD64) but have low levels or lack of important accessory molecules (CD40, CD54, CD58, CD80, CD86), which mediate the binding and stimulation of naive T lymphocytes. To become potent T cell stimulators, DC have to mature. This process is accompanied by phenotypic and functional alterations. Immature myeloid DC, characterized by high expression of CD1a, reside in peripheral tissues. After antigen uptake and exposure to inflammatory stimuli they migrate to peripheral lymph nodes and undergo maturation. During maturation DC lose their ability to efficiently take up and process antigens. The expression of molecules for

potent antigen presentation to T cells (e.g. HLA-DR, CD40, CD86, CD83, CD80) is strongly upregulated.

The differentiation of monocytes either into macrophages or myeloid dendritic cells is antagonistically regulated by cytokines. IL-10 and M-CSF suppress DC differentiation / maturation and enhances CD14 expression as indicator for monocytes / macrophages. IL-4 and GM-CSF promote the differentiation of dendritic cells. DC maturation occurs in vitro by addition of LPS or inflammatory cytokines (e.g. TNF-□). The induction or inhibition of DC maturation is a substantial immune regulatory principle. Promotion of DC maturation leads to enhancement of adaptive immunity whereas its inhibition results in suppression of specific immunity with a simultaneous enhancement of the innate scavenging functions by macrophages without the induction of a specific immune reaction (Alexis et al, 2005; Allavena et al, 1998; Corinti et al, 2001; Fiorentino et al, 1991; Fortsch et al, 2000; Steinbrink et al, 1997; Zeyda et al, 2005).

Figure 3 shows the differentiation and maturation process of myeloid dendritic cells from blood monocytes *in vitro*. The biological process of monocytes differentiation into DC can be imitated by treatment with GM-CSF/IL-4 for 7 days. A following addition of the inflammatory stimulus LPS leads to the maturation of immunocompetent dendritic cells. For the study of the immunoregulatory mechanisms of HO-1 this pathway for generation of myeloid DC was used (Banchereau et al, 2003).

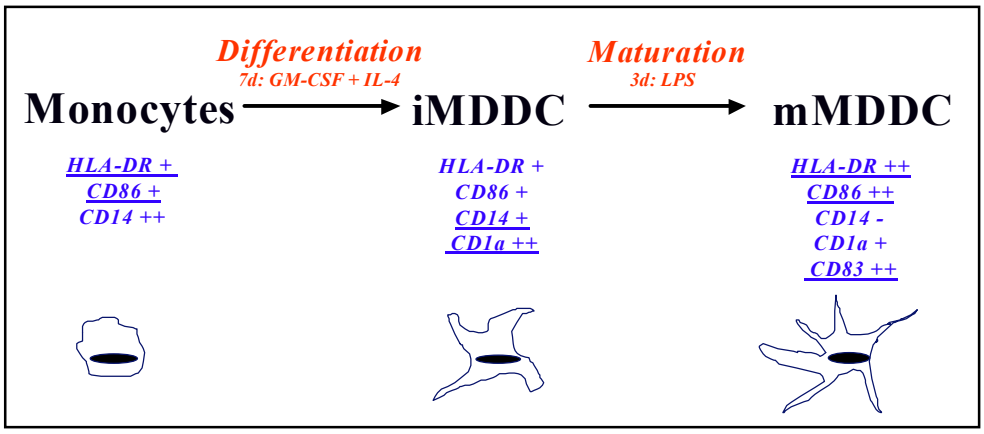


Figure 3. Generation of myeloid DC in vitro
Myeloid DC can be generated *in vitro* from $CD14^+$ monocytes. Combined GM-CSF and IL-4 treatment leads to the differentiation of monocytes into immature dendritic cells (induction of CD1a). Under these conditions generated DC used as an equivalent for *in vivo* immature DC. Stimulation with LPS results in maturation and generation of high immunostimulatory DC with up-regulated motility, costimulatory signals and CD83 expression and down-regulation of antigen-processing machinery.

1.3 Mammalian Skin: an immunologically important organ

Skin is an important immunological organ, which represents the biggest physical barriers against environment. For sufficient defense skin is composed of different immunological active cell types, which are either resident or migrate into the skin dependent on skin conditions (e.g. in skin inflammation).

1.3.1 Skin organization

Integumentum commune is the outermost body surface and consists of cutis (epidermis, dermis) and subcutis (adipose connective tissue). The epidermis serves as the first epithelial barrier against microorganisms and foreign substance and is built by several differentiation stages of keratinocytes (synthesis of intermediate keratins). Other cells in epidermis are melanocytes (for melanin synthesis) and the immunological important Langerhans cells, which represent a subgroup of the myeloid DC (Banchereau et al, 2003) (see also 1.2.1.2.2). Dermis and subcutis are connective tissues with support functions for epidermis (blood vessels, sebaceous and sweat glands, etc.). Dermis contains fibroblasts (synthesis of extracellular matrix) and immune cells like mast cells, macrophages, lymphocytes (Fig.4).

1.3.2 Skin immune reactions

Skin as an important immunological protection system can undergo strong phenotypical and immunological alterations after stimulation of skin immune cells (e.g. challenge in contact allergies) or in autoimmune skin diseases (e.g. Psoriasis). The skin immunoreaction and skin biomarkers can be investigated for drug discovery.

1.3.2.1 Contact hypersensitivity reaction

Contact dermatitis can be divided into two phases: the sensitization and the elicitation (challenge) phase. In the first phase, the skin has for the first time contact with the contact sensitizer and this leads to an allergen-specific immune response without any skin-specific symptoms. The specific allergen is taken up by antigen-presenting cells (Langerhans cells in epidermis), which migrate from sensitized skin into neighboring lymph nodes where they present processed allergen / antigen to specific T cells.
The second contact with the same allergen leads to a specific immune reaction with the typical symptoms of contact dermatitis (i.e. edema and swelling, redness and teleangiectasia, sometimes blisters, cell infiltration). This response is primarily mediated by allergen-specific memory T cells but other cells, e.g. eosinophils, are also involved. The T cell-dependent inflammatory skin reaction is reduced to normal level within several days of antigen elimination.

1.3.2.2 Psoriasis

Psoriasis is a chronic autoimmune disease of unsolved pathogenesis affecting skin (and in a patient subpopulation also joints) of about 3% of the general population. It is characterized by inflamed and disfiguring skin lesions (Fig. 5) and oligoarthritis of the hands and feet. Skin lesions are characterized by epidermal abnormalities (hyperkeratosis, epidermal hyperplasia) (Fig. 4b) and by strong participation of Th1 cells, NK cells, neutrophils, monocytes/macrophages, dendritic cells, proinflammatory cytokines (IFN-γ, TNF-α, IL-8, IL-12, IL-18,), chemokines, Toll-like receptors, anti-microbial peptides (defensins) and complement system (Bos et al, 2005).

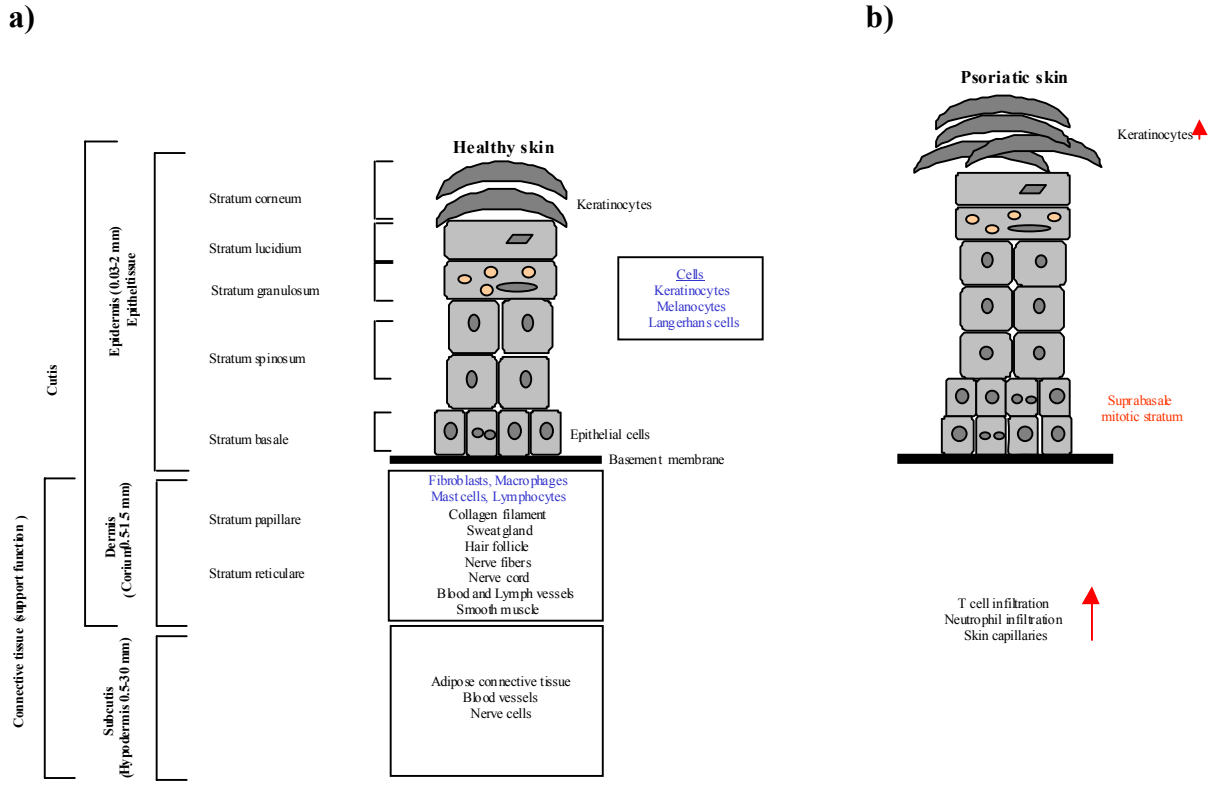


Figure 4. Structure of mammalian skin and psoriasis
The schemes show the cellular architecture of healthy (a) and the changes in epidermis of psoriatic skin (b). Skin is the largest organ composed of two main tissues: the epidermis and the underlying connective tissue, which consists of the dermis and the subcutis. Each tissue is composed of a variety of cell types. The dermis and subcutis are richly supplied with blood vessels and nerves. The epidermis consists of several layers formed by different development stadiums of keratinocytes (a).
The epidermis of psoriatic skin (b) is characterized by hyperproliferation and altered differentiation of epidermal keratinocytes. Strong infiltrates of T cells and neutrophils, increased expression of proinflammatory cytokines and an increase of skin vascularization have also been demonstrated (Schon, 1999; Schon et al, 2005).

Figure 5. Clinical pictures of psoriasis
Severe hyperkeratotic plaque psoriasis (elbow). (b) Generalized pustular psoriasis (erythroderma). (c) Inflammatory type of plaque psoriasis (trunk) (Bos et al, 2005).

1.4 The aim of the study

Heme oxygenase 1 (HO-1) is an antiinflammatory stress protein. Its immunosuppressive activity has been demonstrated in several models of inflammation, autoimmunity and allotransplantation. Whereas the antiinflammatory activity of HO-1 induction is well characterized, the mechanisms of its immunosuppressive effects, i.e. the depression of T cell immunity, are poorly understood.

The principal aims of this work were :

i. Characterization of HO-1 expression in T cell-dependent skin inflammation both in human diseases and in experimental model
ii. Determination of the physiological role of HO-1 in skin inflammation
iii. Investigation of the effect of pharmacological HO-1 induction on skin inflammation
iv. Investigation of the effects of enhanced HO-1 activity on T cell immunity in *ex vivo* and *in vitro* assays
v. Clarification of mechanisms underlying the inhibition of adaptive immunity by investigation of the effects of enhanced HO-1 activity on the differentiation, maturation and function of antigen-presenting cells.

In detail the following investigations were done in clinical and experimental skin inflammation and in *ex vivo* and *in vitro* immunoassays:

1. Determination of HO-1 expression in T cell-dependent skin inflammation
    - 1.1 Acute contact dermatitis in human
    - 1.2 Contact hypersensitivity model in mice
    - 1.3 Ps1.1 oria sis
2. Characterization of the physiological role of HO-1 in experimental skin inflammation in mice by treatment with the HO-1 inhibitor Sn-PPIX
3. Effect of in HO-1 induction on skin inflammation in contact hypersensitivity models in mice
    - 3.1 Effect of the HO-1 inducer Co-PPIX on in vivo HO-1 expression and activity
    - 3.2 Effect of Co-PPIX treatment on skin inflammation in DNFB- and TMA-induced contact hypersensitivity models
4. Effects of HO-1 induction on T cell immunity
    - 4.1 Effect of Co-PPIX treatment in mice on the *ex vivo* alloreactive proliferation of splenocytes
    - 4.2 Effect of HO-1 induction by *in vitro* Co-PPIX treatment on the proliferation of human peripheral blood mononuclear cells in response to allo- and recall antigens
5. Effect of HO-1 induction on differentiation, maturation and function of antigen-presenting cells
    - 5.1 Increase of HO-1 expression in monocytic cells and monocyte-derived dendritic cells (MDDC) by treatment with Co-PPIX or by adenoviral HO-1 transduction
    - 5.2 Effect of enhanced HO-1 expression on the immunophenotype of monocytes and the immunophenotypic differentiation and maturation of MDDC
    - 5.3 Effect of HO-1 induction on the LPS-induced cytokine production of monocytes and MDDC
    - 5.4 Effect of HO-1 induction or adenoviral HO-1 transduction on the antigen-presenting capacity of MDDC in response to allo- and recall-antigens

This work characterizes for the first time the antiinflammatory activity of HO-1 in T cell-dependent skin inflammation. Moreover it determines the inhibition of the differentiation, maturation and function of antigen-presenting cells as an important mechanism for the inhibition of adaptive immunity by HO-1.

2 Materials and methods

2.1 Materials

2.1.1 Equipment and labware

Equipment:

ABI Prism 7900HT Detection System	Applied Biosystems, Darmstadt
ABI Prism 6100 Nucleic Acid PrepStation	Applied Biosystems, Darmstadt
Agilent 2100 Bioanalyzer	Applied Biosystems, Darmstadt
ß-Counter	Wallack, Freiburg
Bio-Plex-Manager-Software	BioRad, München
Bio-Plex Protein Array System	BioRad, München
Cassette (lightproof)	Rego, Augsburg
Cell-Harvester	Packard, Dreieich
Centrifuge	Heraeus Sepatech, Berlin
Chip Priming station	Applied Biosystems, Darmstadt
Cryostat Leica CM3050S	Leica, München
Curix 60	Agfa, Berlin
Dako-Pen	Dako, Hamburg
Destillator Milli-Q biocel	Millipore, Eschborn
Eppendorf-Pipettes	Merck, Berlin
Eppendorf Thermomixer comfort	Merck, Berlin
Epson Perfection 4870 Photo	Epson, Mannheim
FACScan / FACSCalibur	Becton Dickinson, Heidelberg
Gene Amp PCR System 9700	Applied Biosystems, Darmstadt
Hyperfilm ECL	Amersham Biosciences, Mainz
Ice machine	Ziegra, Isernhagen
IKA Ultra Turrax TSFU Homogenizer	IKA, Staufen
IKA Works Vortexer (model MS2-S9)	IKA, Staufen
Immersion oil	Carl Zeiss, Jena
Incubator	Heraeus BBD6220, Berlin
Luminex 100 instrument	BioRad, München
MACS-Stand-Multi	Miltenyi, Biotec, Bergisch Gladbach
Meta Vue	Visitron, Puchheim
Microscope Axiovert 25	Carl Zeiss, Jena

Microscope Axioplan	Carl Zeiss, Jena
Multifuge 3s	Kendro Laboratory products, Heraeus, Berlin
Neubauer cell counting chamber	Brand, Berlin
Nitrocellulose membranes	Invitrogen, Karlsruhe
NuPAGE gel	Invitrogen, Karlsruhe
Octo- and midi-MACS (VS+)	Miltenyi Biotec, Bergisch Gladbach
Polytron PT 3100 homogenizer	Kinematica, Frankfurt am Main
Refrigerator (-20)	Bosch, Berlin
Refrigerator (-80)	Kendro Heraeus, Berlin
RNA chip	Applied Biosystems, Darmstadt
Scale	Sartorius, Göttingen
Schilling cell counting chamber	Schreck, Hofheim
Shaver	Bosch, Berlin
Sorvall RC 5C Plus	ThermoElectroncorporation, Erlangen
Spectral photometer (Spectra Max 340)	Molecular Devices, Heidelberg
Sterile bench	Hera Safe, Berlin
Power supply	Micro Bio Tec Brand, Giessen
Vacuum-Millipore	Millipore, Eschborn
Vortex Titramax 100T	Heidolph, Freiburg
Water bath	Julabo U3, Berlin
X Cell II Blot Module	Invitrogen, Karlsruhe
X Cell Sure-Lock Electrophoresis Cell	Invitrogen, Karlsruhe

Labware:

BioPlex Filter plate	BioRad, München
Cell culture flask	Costar, Bodenheim
Cell strainer 70 µm	Becton Dickinson, Heidelberg
Collection Plate (2 mL)	Applied Biosystems, Darmstadt
Costar 6 well plate	Costar, Bodenheim
Costar 48 well plate	Costar, Bodenheim
Costar 96 well plate	Costar, Bodenheim
Costar 96 round well plate	Costar, Bodenheim
Cover slip	Menzel, Wiesbaden
Eppendorf-Tube (0.5 mL)	Eppendorf, Hamburg
Eppendorf-Tube (1 mL)	Eppendorf, Hamburg
Falcon 5 mL tube	Becton Dickinson, Heidelberg

Falcon 6 well plate	Becton Dickinson, Heidelberg
Falcon PP 14 mL	Becton Dickinson, Heidelberg
Filter	Millipore, Eschborn
Filter-Plate	Packard, Dreieich
Large Volume Adapter Plate	Applied Biosystems, Darmstadt
Large Volume RNA Prep Filter	Applied Biosystems, Darmstadt
LeucoSep tube	Greiner, Frickenhausen
LS+ separation column	Miltenyi Biotec, Bergisch Gladbach
MicroAmp Optical Caps	Applied Biosystems, Darmstadt
Microcentrifuge tube (2 mL)	Applied Biosystems, Darmstadt
Micronic tubes (1 mL)	R&D Systems, Wiesbaden
Micropipettes	Eppendorf, Hamburg
Monovette	Sarstedt, Nümbrecht
Needles	Braun, Melsungen
Pipettes (sterile)	Becton Dickinson, Heidelberg
Polypropylene tubes	Sarstedt, Nümbrecht
Reservoir 5 mL	Applied Biosystems, Darmstadt
Reservoir 20 mL	Applied Biosystems, Darmstadt
RNA-Purification Trays	Applied Biosystems, Darmstadt
Syringes	Braun, Melsungen
S-Monovette 9 NC	Sarstedt, Nümbrecht
S-Monovette LH	Sarstedt, Nümbrecht
Splash Guards	Applied Biosystems, Darmstadt
SuperFrost plus (Object holder)	Menzel, Wiesbaden
Tissue Culture Dish (100 mm)	Costar, Bodenheim
Vacuette Tempus Blood RNA Tube	Applied Biosystems, Darmstadt

2.1.2 Medium, buffers and chemicals

Medium, Buffers, Solutions:

Absolute RNA Wash Solution	Applied Biosystems, Darmstadt
BCA protein assay	Pierce, Berlin
Cell Lysing Solution	Applied Biosystems, Darmstadt
DNAse/RNAse free water	Gibco BRL, Eggenstein
ECL kit	Amersham Biosciences, Mainz
FACSFlow solution	Becton Dickinson, Heidelberg
FCS	Gibco BRL, Eggenstein
Faramount (watery medium)	Dako, Hamburg
Ficoll-Plaque TM Plus	Amersham Biosciences, Mainz
Histopaque-1077 separation solution	Sigma, Deisenhofen
Invisorb RNA Kit II	Invitek, Berlin
Nucleic Acid Purification Elution Solution	Applied Biosystems, Darmstadt
NuPAGE LDS sample buffer	Invitrogen, Karlsruhe
NuPAGE running buffer	Invitrogen, Karlsruhe
NuPAGE transfer buffer	Invitrogen, Karlsruhe
Olive oil	Sigma, Deisenhofen
PBS (without Ca^{2+} / Mg^{2+})	Gibco BRL, Eggenstein
RNAse-free water	Gibco BRL, Eggenstein
RNA 6000 Nano Assay kit	Ambion Biotechnology, Cambridgeshire, UK
RNA-Purification Wash solution 1	Applied Biosystems, Darmstadt
RNA Purification Wash Solution 2	Applied Biosystems, Darmstadt
Scot´s tape water	Sigma, Deisenhofen
TaqMan 10x RT Buffer (No AmpErase UNG)	Applied Biosystems, Darmstadt
Tissue freezing medium (Tissue tec)	Leica, München
TRIS-buffer (0.001M)	Merck, Darmstadt
Universal PCR Master Mix	Applied Biosystems, Darmstadt
VLE RPMI 1640 medium	Biochrom, Berlin

Chemicals:

Acetone	Merck, Darmstadt
AEC (3-amino-9-ethylcarbacol)	Pharmingen, Heidelberg
AMC (7-amino-4-methyl-coumarin)	Sigma, Deisenhofen
Bilirubin reagent	Sigma, Deisenhofen
Bilirubin standard	Sigma, Deisenhofen
Bio-Plex Cytokine Assay	BioRad, München
Bovine serum albumin (BSA)	Sigma, Taufkirchen
Cetrimid	Sigma, Deisenhofen
CFDA-SE (Carboxyfluorescein diacetate succinimidyl ester)	Molecular Probes, München
Chloroform	Merck, Darmstadt
CO_2	Linde, Höllriegelskreuth
Complete (Mini, EDTA free)	Roche, Mannheim
Deoxy-NTPs Mixture	Applied Biosystems, Darmstadt
Dimethylsulfoxide (DMSO)	Merck, Darmstadt
Dithiothreitol (DTT)	Gibco BRL, Eggenstein
DMDM	Sigma, Deisenhofen
EDTA	Sigma, Deisenhofen
EGTA	Sigma, Deisenhofen
Eosin	Dako, Hamburg
Ethanol	Merck, Darmstadt
Fast green stain (FW 808.8)	Sigma, Deisenhofen
FSB (5x)	Invitrogen, Karlsruhe
Glycogen (35 mg/mL)	Invitrogen, Karlsruhe
HCl	Merck, Darmstadt
Hematoxylin	Dako, Hamburg
H_2SO_4	Sigma, Deisenhofen
HTAB (Hexadecyltrimethylammoniumbromid)	Sigma, Deisenhofen
Isopropanol	Invitek, Berlin
Isopropylmyristate	Sigma, Deisenhofen
KCl	Sigma, Deisenhofen
Ketavet 10 mg/mL (Ketaminhydrochlorid)	Pharmacia, Erlangen
$KHCO_3$	Sigma, Deisenhofen
Liquid nitrogen	Linde, Höllriegelskreuth
Magnesium Chloride 25 mM ($MgCl_2$)	Applied Biosystems, Darmstadt
Mec-Succ-Ala-Ala-Pro-Val-AMC (substrate)	Bachem AG, Weil am Rhein

Mitomycin	Sigma, Deisenhofen
MMLV (Moloney Murine Leukemia, Virus)	Invitrogen, Karlsruhe
MOPS (3-N-Morpholino propanesulfonic acid)	Sigma, Deisenhofen
MultiScribe Reverse Transcriptase (50 U/µL)	Applied Biosystems, Darmstadt
NaCl	Sigma, Deisenhofen
Na_2CO_3	Sigma, Deisenhofen
NaH_2PO_4	Merck, Darmstadt
NaN_3	Merck, Darmstadt
NaOH	Merck, Darmstadt
NH_4Cl	Sigma, Deisenhofen
NuPAGE Antioxidant	Invitrogen, Karlsruhe
NuPAGE reducing agent	Invitrogen, Karlsruhe
OdT-Primer: pd (T) 12-18	Amersham, Mainz
Penicillin-Streptomycin	Gibco BRL, Eggenstein
Perdrogen	Sigma, Deisenhofen
Peroxidase stop	Dako, Hamburg
Phenol (RNA / DNA Isolation)	Roth, Karlsruhe
Phosphatase inhibitor cocktail I	Sigma, Deisenhofen
Phosphatase inhibitor cocktail II	Sigma, Deisenhofen
Propidiumiodide	Sigma, Deisenhofen
Proteinase K	BD Biosciences, Heidelberg
Random Hexamers	Applied Biosystems, Darmstadt
RNAse Inhibitor	Applied Biosystems, Darmstadt
RNA 6000 Ladder (150 ng/µL)	Ambion Biotechnology, Cambridgeshire, UK
Scintillator	Wallack, Heidelberg
Sea blue protein ladder Plus 2	Invitrogen, Karlsruhe
Sodium acetate	Sigma, Deisenhofen
Sodium nitrite solution	Sigma, Deisenhofen
Streptavidin-HRP	Pharmingen, Heidelberg
TMB	Sigma, Deisenhofen
[^{3}H]-thymidine	Amersham, Mainz
Titrisol (H_2SO_4)	Merck, Darmstadt
Trimerosal	Merck, Darmstadt
Tris	Merck, Darmstadt
Triton X-100	Merck, Darmstadt
Trypan blue	Serva, Heidelberg

Tween 20 (Polyoxyethylene sorbitan monolaurate)	Sigma, Deisenhofen

2.1.3 HO-1 inducer/inhibitor, stimulators, cytokines, antibodies

HO-1 inducer / inhibitor:

Co-PPIX	Sigma, Deisenhofen
Sn-PPIX	Sigma, Deisenhofen

Haptens, Stimulators, Cytokines:

DNFB	Sigma, Deisenhofen
LPS (*E. coli* strain 0127:B8)	Sigma, Deisenhofen
rhGM-CSF	R&D Systems, Wiesbaden
rhIFN-γ	R&D Systems, Wiesbaden
rhIL-4	R&D Systems, Wiesbaden
rhIL-10	R&D Systems, Wiesbaden
rhIL-12	R&D Systems, Wiesbaden
rhM-CSF	R&D Systems, Wiesbaden
Tetanus toxoid (0.08 IU/mL)	Smith Kline Beecham Pharma, München
TMA	Sigma, Deisenhofen

Antibodies:

Antibodies for stimulation, WB, histology:

Anti-CD3 (OKT3)	Cilag, Sülzbach
Anti-CD4	Pharmingen, Heidelberg
Anti-CD8	Pharmingen, Heidelberg
Anti-CD28	R&D Systems, Wiesbaden
Anti-IFN-γ	R&D Systems, Wiesbaden
Anti-HO-1 (rabbit)	Biotrend , Köln
Anti-I-A/I-E	BD Biosciences, Heidelberg
Anti-IL-4	R&D Systems, Wiesbaden
Anti-Neutrophils	Acris, Hiddenhausen
Anti-rabbit peroxidase conjugated antibody	Dianova, Hamburg
Antiserum goat	Dako, Hamburg
Goat anti rabbit	Dako, Hamburg
Goat anti rat	Pharmingen, Heidelberg

Antibodies for Flow Cytometry:

CD3-FITC	Beckman Coulter, Krefeld
CD4-FITC	Beckman Coulter, Krefeld
CD8-FITC	Beckman Coulter, Krefeld
CD14-FITC	Beckman Coulter, Krefeld
CD19-FITC	Beckman Coulter, Krefeld
CD83-FITC	Beckman Coulter, Krefeld
CD1a-PE	Beckman Coulter, Krefeld
CD4-PE	Beckman Coulter, Krefeld
CD8-PE	Beckman Coulter, Krefeld
CD86-PE	Becton Dickinson, Heidelberg
HLA-DR-PE	Becton Dickinson, Heidelberg
CD3-PerCP	Becton Dickinson, Heidelberg
CD4-PC5	Beckman Coulter, Krefeld
CD4-PerCP-Cy5.5	Becton Dickinson, Heidelberg
CD14-PC5	Beckman Coulter, Krefeld

2.1.4 Kits for cell separation, cell function and cytokine detection

Cell separation:

$CD4^+$ T cell isolation kit (MACS)	Miltenyi Biotec, Bergisch Gladbach
$CD8^+$ T cell isolation kit (MACS)	Miltenyi Biotec, Bergisch Gladbach
T-Cell Enrichment Cocktail (RosetteSep)	StemCell Technologies, Vancouver, Canada
Monocyte isolation kit (MACS)	Miltenyi Biotec, Bergisch Gladbach
Monocyte Enrichment Cocktail (RosetteSep)	StemCell Technologies, Vancouver, Canada

Cell function and apoptosis tests:

Annexin V-FITC apoptosis detection kit	BD Biosciences, Heidelberg
Burst test	Orpegen Pharma, Heidelberg
Phagocytosis test	Orpegen Pharma, Heidelberg

ELISAs:

hIL-2	R&D Systems, Wiesbaden
hIL-10	Biosource, Belgium
hIL-12	R&D Systems, Wiesbaden
hIL-12p40	R&D Systems, Wiesbaden
hIL-18	MBL, Everswinkel
hTNF-α	Biosource, Belgium
HO-1 ELISA	Stressgen, Hamburg
Mouse IL-4 Minikit	Endogen, Eching

2.1.5 Primers and probes for real-time RT-PCR (TaqMan)

Primers	Tib Molbiol, Berlin
Probes	Metabion, Planegg
Assays on Demand	Applied Biosystems, Darmstadt

Mouse HO-1:

Forward primer	5`-CAGAAGAGGCTAAGACCGCCTT -3´	300 nM
Reverse primer	5`-TCTGGTCTTTGTGTTCCTCTGTCA -3´	300 nM
Probe	5´-TGCTCAACATTGAGCTGTTTGAGGAGCTG-3´	200 nM

Mouse Beta-actin:

Forward primer	5´-GTACAACCTCCTTGCAGCTCCT -3´	300 nM
Reverse primer	5`-TTGTCGACGACGAGCGC -3´	900 nM
Probe	5´-CGCCACCAGTTCGCCATGGAT -3´	200 nM

Human HO-1:

Forward primer	5‘- GAAGAGGCCAAGACTGCGTTC-3‘	50 nM
Reverse primer	5‘-TGGTCCTTGGTGTCATGGGT-3‘	300 nM
Probe	5‘-TGCTCAACATCCAGCTCTTTGAGGAGTTG-3‘	200 nM

Human HPRT:

Forward primer	5‘-AGTCTGGCTTATATCCAACACTTCG-3‘	300 nM
Reverse primer	5‘-GACTTTGCTTTCCTTGGTCAGG-3‘	300 nM
Probe	5‘-TTTCACCAGCAAGCTTGCGACCTTGA-3‘	200 nM

2.1.6 Human biomaterial and mice

Biomaterial from healthy control probands and from patients with psoriasis or nickel allergy was obtained from the Interdisciplinary Group of Molecular Immunopathology, Dermatology / Medical Immunology, University Hospital Charité (Berlin, Germany). The obtained skin was about 1 cm^2 in size and about 0.3 mm thick.
Blood from healthy male volunteers was taken off in Institute of Clinical Pharmacology at Schering AG.

NMRI outbred- and C57Bl/6 and Balb/c inbred-mice weighing 24-26 g were purchased from Charles River (Berlin). At the time of testing, mice were between the ages of 8 and 12 weeks.

All human and animal studies were approved by the competent authority for labor protection, occupational health, and technical safety for the state and city of Berlin, Germany, and were performed in accordance with the ethical guidelines of Schering AG.

2.2 Methods

2.2.1 Preparation of synthetic metalloporphyrins

Cobaltic (Co-PPIX) and tin (Sn-PPIX) protoporphyrin IX chloride are synthetic metalloporphyrins. Whereas Co-PPIX is a well known specific inducer of heme oxygenase 1 (HO-1) expression and activity, Sn-PPIX is a specific inhibitor of HO-1 enzyme activity.

Co-PPIX (MW 655 g/Mol) and Sn-PPIX (MG 626 g/Mol) were dissolved in 0.1 M NaOH in sterile 5 mL-Falcon tubes. For *in vivo* assays Co-PPIX and Sn-PPIX solutions were adjusted to pH 7.4 with HCl and further diluted in NaCl. For *in vitro* assays, in NaOH dissolved Co-PPIX was diluted with 0.01 M NaH_2PO_4 and FCS up to 500 µM stock concentration. All further dilutions occurred in the vehicle adequate to the highest Co-PPIX concentration.

Example for Co-PPIX preparation for in vitro tests:

1)

16.3 mg Co-PPIX
+ 1000 µL 0.1 M NaOH

= 25 mM Co-PPIX solution

2)

50 µL of 25 mM Co-PPIX solution
+ 1200 µL 0.01 NaH_2PO_4
+ 1250 µL 100% FCS

= 500 µM Co-PPIX solution

Example for Co-PPIX (Sn-PPIX) preparation for in vivo tests in mice:

30 mg/kg required per 24 g weight

1)

30 mg/1000g = 0.72 mg/24 g mouse
0.72 mg application in 100 µL volume per mouse

2)

solution in about 50 µL NaOH
+ HCl for adjustion (pH 7.4)
+ fill up to 100 µL volume with NaCl

= 0.72 mg Co-PPIX in 100 µL volume per 24 g

2.2.2 Animal models

2.2.2.1 HO-1 gene expression in mice organs

To investigate *in vivo* HO-1 mRNA induction, Balb/c mice were treated i.p. with 15 mg/kg Co-PPIX (0.2 mL). After 6 hours, mice were sacrified by cervical translocation, the skin was disinfected with 70% ethanol, and organs were extracted, snap-frozen in liquid nitrogen in polypropylene tubes and stored at –80°C until use. After homogenization in 800 µl lysis buffer (Applied Biosystems) and digestion with 2 mg/mL Proteinase K for 1 hour, HO-1 mRNA expression in mouse organs was determined by the classical quantitative real-time RT-PCR with primers and probes for mouse HO-1 and beta-actin (see 2.2.4.9.1).

2.2.2.2 Systemic bilirubin levels as parameter for HO-1 activity in mice

Balb/c mice (three mice per group) were treated i.p. with 20 mg/kg Co-PPIX (0.2 mL) for one to three days. Mice were anesthetized with 0.3 mL Ketavet i.p. (1:3 in NaCl), and 800-1000 µL whole blood was taken up with a 0.9-gage needle from the *vena cava inferior* (below liver) in 2 mL Eppendorf tubes. After centrifugation (300 x g, 10 min, RT) about 300 µL serum was collected for bilirubin measurement (see 2.2.4.5.5).

2.2.2.3 Ex vivo mixed leukocyte reaction (MLR) as parameter for immunosuppression

Mice (C57Bl/6, Balb/c) were treated i.p. over 24 hours with 20 mg/kg Co-PPIX or vehicle and, after killing by cervical dislocation, spleens were taken out, splenocytes were separated (see 2.2.4.1.4) and applicated for MLR (2.2.4.4.1.2).

2.2.2.4 Contact hypersensitivity models

Background

Allergic contact dermatitis or contact hypersensitivity (CHS) can be divided into two phases: the sensitization and the elicitation (challenge) phase. In the first phase, the animals have the first contact (ears, flank) with the contact sensitizer. This leads to an allergen-specific immune response - called sensitization without any skin-specific symptoms - and to the priming of allergen/hapten-specific T cells by antigen presenting cells which have uptaken the allergens and are migrated from sensitized skin into the neighboring lymph nodes. The second skin contact of the animal with the same allergen

leads to a specific immune reaction with the typical symptoms of contact dermatitis (i.e. edema and swelling, redness and teleangiectasia, sometimes blisters, cell infiltration). This response is mediated primarily by allergen-specific memory T cells but other cells, e.g. neutrophils and eosinophils, are also involved. Quantification of the elicitation phase (or challenge response) is routinely performed 24 hours after the challenge. Standard parameters for quantifying the inflammatory response are measurements of ear thickness or ear weight. Increases in these parameters are caused by plasma extravasation and by cellular infiltration (lymphocytes, macrophages, neutrophil granulocytes). Cellular infiltration can be quantified by histology or by measuring cell-specific enzymes such as peroxidase (specific for myeloid cells) and PMN-elastase (specific for neutrophils).

2.2.2.4.1 2,4-Dinitrofluorobenzene (DNFB)-induced allergic contact dermatitis

This murine contact hypersensitivity model represents a T cell-dependent model of skin inflammation characterized by a dominant Type 1 cytokine expression profile.

DNFB-induced ear inflammation model

NMRI mice were sensitized on day 0 and 1 with 25 µl 0.5% DNFB dissolved in 80:20 acetone/olive oil on shaved abdomens (1 cm^2). On day 5 mice were challenged by applying 20 µl of 0.3% DNFB (w/v) onto the dorsal sides of both ears. Intraperitoneal Co-PPIX treatment was either performed before / around sensitization (d0 or d0-d3) or at 6 hours before challenge. Sn-PPIX treatment was done either at 2 hours or at 6 hours before challenge. At 24 hours after challenge (plateau of inflammation) mice were sacrified (CO_2) and the ears were cut off and weighed as a measurement for edema formation. Additionally, ears (without Co-PPIX treatment) were snap frozen in Tissue freezing medium (Tissue tec) for immunohistology (see 2.2.4.8, 2.2.4.8.1, 2.2.4.8.2).

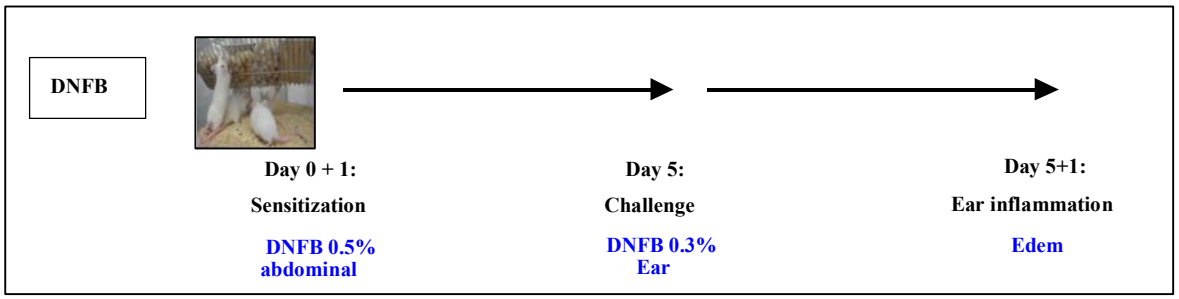


Figure 6. DNFB-induced CHS model in mice
Mice were sensitized on day 0 and 1 with DNFB, challenged four days later and at 24 hours after challenge the inflammation was measured.

Determination of HO-1 mRNA expression in DNFB-challenged flank skin

For determination of HO-1 expression, skin samples (0.5 cm^2, 0.2 mm thick, 10-30 mg) were yielded either after DNFB sensitization before challenge or at 1, 3, 6, 24, and 48 hours after DNFB challenge. The skin was snap-frozen in liquid nitrogen in polypropylene tubes and stored at –80°C until use. After homogenization in 800 µl lysis buffer (Applied Biosystems) and digestion with 2 mg/mL Proteinase K for 1 hour, total RNA and cDNA were prepared with the classical methods (2.2.4.9.1). Mouse HO-1 and HPRT mRNA was determined with AoDs (2.2.4.9.2).

2.2.2.4.2 Trimellitic anhydride (TMA) allergic contact dermatitis

Sensitization with the contact allergen trimellitic anhydride (TMA) induces a delayed-type hypersensitivity (DTH) reaction, with prominent eosinophil infiltration at 24 hours after challenge, a significant increase in serum IgE concentrations, and a Type 2 cytokine expression profile (Dearman et al, 1996).

TMA-induced ear inflammation model

NMRI mice were sensitized on day 0 by application of 50 µl of 3% (w/v) TMA in acetone/isopropylmyristate (80/20) onto a shaved area of abdominal skin. The DTH reaction was induced on day 5 by challenging the animals with 10 µl of 3% (w/v) TMA in acetone/isopropylmyristate (80/20) onto the dorsal sides of both ears. Intraperitoneal application of Co-PPIX (0.2 mL) was carried out at 1 day before challenge. At 24 hours after challenge (at maximum of the inflammatory response) mice were sacrified (CO_2), and the ears were cut off and weighed as a measurement for edema formation and frozen in liquid nitrogen in polypropylene tubes for determination of IL-4 levels.

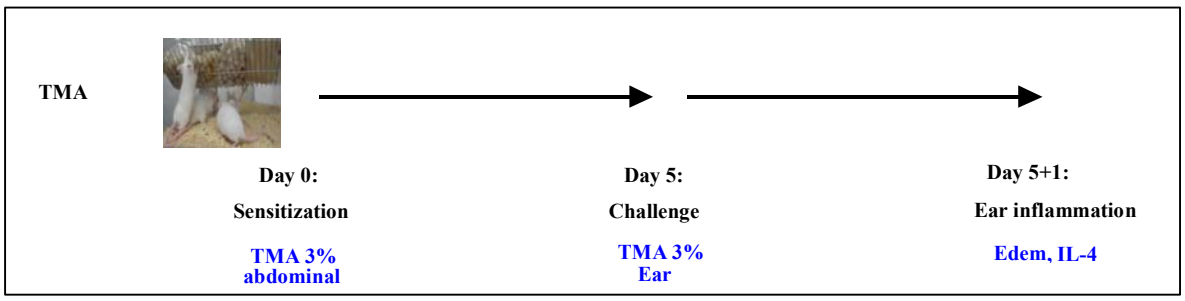


Figure 7. TMA-induced CHS model in mice
Mice were sensitized on day 0 with TMA, challenged five days later and at 24 hours after challenge the inflammation was determined.

Ear preparation for IL-4 detection in tissue homogenates

Thawed ears were homogenized in 2 mL homogenate buffer under constantly cooling with ice-cold 40% ethanol for 20 second in a Polytron® PT 3100 homogenizer (Kinematica) set to the maximum speed (30,000 rpm). Thereafter, samples were centrifuged at 24,000 x g for 20 minutes at 10°C, and 750 µL of supernatants of the aqueous homogenates were transferred into 96-deep-well-plates and stored at –20°C. The supernatants are then used for determination of IL-4 as an indicator of Th2 immune response (see 2.2.4.5.4).

Homogenate buffer (1 L), pH 7.0:

0.5% Hexadecyltrimethylammoniumbromid (HTAB) (=5 g)
10 mM MOPS (=2.09 g)
Both chemicals were dissolved at 30-35°C in 900 mL aqua bidest, neutralized with 1N NaOH and fill up to 1 L with aqua bidest.

2.2.3 Statistical analysis for in vivo experiments

Statistical analysis was performed with the so-called „modified Hemm“ (inhibition) test based on Fieller´s test from SAS system for Windows 6.12 (SAS Institute Inc.) (Schottelius et al, 2002). To determine the inhibitory effect of anti-inflammatory compounds, the difference between the respective mean value of the positive controls and the mean value of the vehicle controls was set to 100% and the percentile change by the test substance was estimated:

$$\% \text{ change} = \frac{\text{Mean value}_{\text{treated group}} - \text{mean value}_{\text{Positive group}}}{\text{Mean value}_{\text{Positive group}} - \text{mean value}_{\text{control group}}} \times 100$$

Figure 8. Formula for calculation of the inhibitory effect of anti-inflammatory compounds.

In order to test whether the change caused by the treatment is different from zero, a 95% confidence interval is calculated under consideration of the variance of observations within the entire experiment. If the interval does not include zero, the hypothesis that there is no change is rejected at the level of $\alpha = 0.05$.

2.2.4 In vitro and ex vivo methods

2.2.4.1 Isolation of cells

2.2.4.1.1 Preparation of human peripheral blood mononuclear cells (PBMC) from peripheral venous blood

Venous blood was taken from healthy voluntary donors with sterile 10 mL-S-Monovette containing 1 mL 0.106 M trisodium (Sarstedt). The required blood amount is dependent on the actual question of the investigation. Usually from 10 mL blood about 1 x 10^7 PBMCs can be isolated by density gradient centrifugation. Briefly, 15 mL Histopaque was filled in 50 mL Leuco-Sep tubes and centrifuged for 1 min at 400 x g and RT. Twenty-five milliliters blood were transferred and centrifuged for 15 min at 800 x g and RT. The upper layer containing plasma and thrombocytes was aspirated. The layer containing the mononuclear cells and the lower Histopaque were transferred into a new 50 mL conical tube. Per 50 mL tube two Leuco-Sep tube contents were united, 30 mL PBS w/o Ca^{2+} and Mg^{2+} was added, and the suspension was centrifuged at 300 x g for 10 min (washing). In order to remove the thrombocytes (as TGF-β source) and Histopaque this washing was repeated three times. After the last washing, pellet was resuspended in either 1 mL VLE RPMI 1640 medium for a PBMCs assay or in 1 mL cold (4-8°C) MACS buffer for further cell isolation. The isolated PBMCs were counted.
Cell counting was performed in a Neubauer´s cell counting chamber by mixing of 20 µl of the PBMC suspension with 200 µl of 0.4% Trypan blue (dissolved in PBS w/o Ca^{2+} and Mg^{2+}) and 180 µl VLE RPMI 1640-Medium in a 1 mL Eppendorf-Tube. This relations corresponding 1:20 dilution and are empirical. Trypan blue is a vital stain. Living cells remove the passive invaded marker actively from cytoplasm whereas dead cells are stained.

Counting of cells at 10 x extension under Axiovert Microscope

Neubauer´s cell counting chamber:
Summation of four quadrates (à16 quadrates) / 4 * 10^4 cells * dilution factor (20)
= cell count per mL

Schilling cell counting chamber:
Summation of four quadrates (à 25 quadrates) / 4 * 10^4 cells * dilution factor (20)
= cell count per mL

After the cell counting, the PBMCs were used in cell culture or applicated for further cell separation.

Medium preparation:

500 mL VLE RPMI 1640 medium
(NaHCO3 and Glutamate)
+ 10% heat inactivated FCS (50 mL)
+ 50 µg/mL Penicillin / Streptomycin

MACS buffer preparation:

500 mL PBS w/o Ca^{2+} and Mg^{2+}
+ 10% heat inactivated FCS (50 mL)
+ sterile filtration

FCS heat inactivation (complement inactivation):

65°C over 30 min

2.2.4.1.2 Negative isolation of cell populations from PBMC by MACS separation

In order to investigate unchanged cell functions, negative (desired cells are unlabeled) cell separation was chosen. After cell counting PBMCs were washed once with ice cold MACS buffer in a 50 mL tube at 10 min and 300 x g at 4°C. All further steps were done in accordance to the protocol of the kit manufacturer's *Miltenyi Biotec* for negative cell separation.

Principle of negative cell separation with MACS

The negative cell isolation is based on depletion of non-desired cells after indirectly magnetically labeling. The desired cells remain unlabeled.
Briefly, addition of an hapten-antibody-cocktail leads to labeling of the non-relevant cells with hapten-conjugated monoclonal antibodies (primary antibodies) directed against various CD antigens. Application of MACS-Anti-Hapten MicroBeads (secondary antibodies) labels indirectly the non-desired cells with paramagnetic beads conjugated with monoclonal antibodies towards the hapten. During separation all paramagnetically labeled cells remain in the LS+ / VS+ column in magnet field. The column capacity is 1 x 10^8 labeled cells from a total of max. 2 x 10^9 cells. All cells in eluate are desired unlabeled cells, which can be counted, analysed for purity and tested in assays after once washing (300 x g, 10 min, RT) in RPMI 1640 cell culture medium.
The following methods for monocytes, $CD4^+$, $CD8^+$ and $CD3^+$ T cell separations (MACS) based on the above described principle and were used for all assays with exception of cell separation from psoriatic whole blood.

Monocyte separation

The monocyte isolation kit consists of three components: hapten-antibody-cocktail, anti-hapten-MicroBeads and FcR-blocking reagent (human immunoglobulins). The blocking reagent protects the Fc-gamma receptors on monocytes against the non-specific binding of the primary and secondary antibodies. By addition of the hapten-antibody-cocktail and anti-hapten-MicroBeads all cells others than monocytes (T, B and NK cells, dendritic cells, thrombocytes, erythrocytes) are indirectly paramagneti-

cally labeled and can be depleted. The expected yield from 1 x 10^8 human PBMC is about 0.25 x 10^8 monocytes in a purity of 90-95%.

CD4$^+$, CD8$^+$ and Pan-T cell separation

The negative T cell isolation kits consist of hapten-antibody-cocktails and the anti-hapten-MicroBeads. All cells except the T-helper cells, the cytotoxic T cells or the Pan-T cells, respectively, are magnetically depleted. The following yields can be expected from 1 x 10^8 human PBMCs in a purity of about 95%.: 0.5 x 10^8 CD4$^+$ T cells; 0.25 x 10 CD8$^+$ T cells; 0.75 x 10^8 CD3+ T cells.

2.2.4.1.3 RosetteSep: A negative isolation of cell populations from whole blood

In order to isolate cell populations directly from EDTA whole blood in shortest time and without magnets and columns the RosetteSep method from StemCell Technologies was used.

Principle of negative separation by RosetteSep

The principle based on the StemCell´s tetrameric antibody complex (TAC), which crosslinks unwanted cells (anti-cell) to multiple red blood cells (anti-glycophorin A) and consequently forms immunorosettes with strongly increased density, which are pelleted by centrifugation over Ficoll. The wanted cells are untouched and highly enriched between the density medium and the plasma.
All separation steps were in accordance with the protocol of StemCell Technologies.
Briefly, whole blood in 9 mL EDTA Monovette was mixed with Enrichment Cocktail (50 µL per 1 mL whole blood) and after 20 min incubation at RT blood were diluted with an equal volume of dilution buffer. Diluted whole blood was overlaid carefully on an equal volume of Ficoll and centrifuged without brake at 1200 x g over 20 min at RT. Separated cells were collected, washed twice with PBS (with 2% FCS) at 300 x g for 10 min at RT. For HO-1 gene expression analysis in separated peripheral blood immune cells, 1 x 10^6 separated cells were lysed in 600 µL cell lysis buffer 1:2 diluted with PBS without Ca^{2+} and Mg^2 (Applied Biosystems).

Dilution buffer

500 mL PBS	
+ 1 mM EDTA (MW 372 g/mol)	0.186 g
+ 2% FCS	10 mL

Monocyte separation

The Monocyte Enrichment Cocktail (RosetteSep) contains a combination of mouse and rat monoclonal antibodies, which are bound in bispecific TAC and directed against human hematopoietic cells (CD2, CD3, CD8, CD19, CD56, CD66b) and glycophorin A on red blood cells. The yielded enriched monocytes (purity about 75%) were further processed for analysis of HO-1 mRNA expression.

T cell separation

In the T-Cell Enrichment Cocktail (RosetteSep) the mouse and rat monoclonal antibodies bound in bispecific TAC are directed against human hematopoietic cell surface antigens (CD16, CD19, CD36, CD56) and glycophorin A on red blood cells. The separated T cells (purity about 95%) were processed for determination of HO-1 expression.

2.2.4.1.4 Isolation of splenocytes from mouse spleen

Mice (NMRI, Balb/c) were killed by cervical dislocation, and their abdominal skin was disinfected with 70% ethanol. The spleen was removed as sterile as possible and transferred into sterile 10 mL of PBS w/o Ca^{2+} and Mg^{2+} at RT. The spleen connective tissue capsule was carefully emptied using two sterile tweezers. The spleen tissue was hacked with sterile scissors and was filtered through a 70 µm cell strainer and then centrifuged for 10 min at 4°C and 300 x g in a 50 mL conical tube. The supernatant was drained off, and the remaining cell pellet was resuspended in 10 mL of erythrocyte lysis buffer and incubated for 3 min at 37°C. Then 40 mL PBS w/o Ca^{2+} and Mg^{2+} were added and centrifuged for 10 min at 4°C and 300 x g. The cells were washed twice with PBS w/o Ca^{2+} and $Mg^{2+}$and were then resuspended in RPMI 1640 cell culture medium and counted.

Erythrocyte lysis buffer:

Aqua bidest	1L
NH_4Cl	8.26 g
$KHCO_3$	1 g
EDTA	0.037 g

2.2.4.2 Generation of monocyte-derived dendritic cells (MDDC) and macrophages from monocytes

The principle of this method is based on the cytokine-triggered orientation of monocyte differentiation either to macrophages (potent phagocytes and scavenger cells) or to monocyte-derived dendritic cells (potent antigen-presenting cells). The generation of immature dendritic cells is done usually by GM-CSF and IL-4 treatment, and the differentiated cells are characterized by high CD1a expression.

Maturation of dendritic cells can be initiated by inflammatory stimuli like LPS, and the cells show high expression of HLA-DR and accessory molecules (CD40, CD80, CD86) as well as of the dendritic cell maturation antigen CD83. Incubation of monocytes with M-CSF leads to the development of macrophages, which do not express CD1a and CD83 but as potent scavenger cells are defined by strong expression of CD14 (LPS/LBP receptor) and e.g. CD16 (Fc-γ receptor 3).

For experiments with monocyte-derived dendritic cell (MDDC), human peripheral blood mononuclear cells (PBMC) were isolated from citrated venous blood of healthy donors by Histopaque-1077 density gradient centrifugation (see 2.2.4.1.1) and monocytes were negatively separated by MACS technique (see 2.2.4.1.2).

2.2.4.2.1 Differentiation of immature MDDC and macrophages

Immature MDDC and macrophages, both 1 x 10^6 cells/mL, were differentiated from monocytes by 7 day-culture with GM-CSF and IL-4 or with M-CSF (all at 10 ng/mL) in 6-well plates in supplemented VLE RPMI 1640 medium. In order to test the effects of HO-1 induction on dendritic cells differentiation, the strong HO-1 inducer Co-PPIX was added to the differentiation culture. Medium including its supplements and Co-PPIX were refreshed (50%) at days 3 and 5 of differentiation culture. After seven day culture at 37°c, 5.0% CO_2 and 95.0% rH in incubator, cells were resuspended and 100 µL (1 x 10^5 cells) were transferred into micronic tubes and labeled with monoclonal antibodies against CD14-FITC, HLA-DR-PE, CD86-PE, CD1a-PE and CD83-FITC. The effect of Co-PPIX on immunophenotypic DC differentiation was measured with FACScan and analysed with CellQuest™ software (Becton Dickinson).

2.2.4.2.2 Maturation of MDDC

Mature MDDC were produced by stimulating of immature MDDC with LPS. Therefore immature DC (not treated with Co-PPIX) were washed twice with VLE RPMI 1640 medium at 250 x g for 10 min at RT, and 1 x 10^6 cells/mL were cultured with 100 ng/mL LPS from *E. coli* 0127:B8 for 3 days in the presence or absence of Co-PPIX. On day three cells were washed with FACS buffer, labeled with the same antibodies as immature DC and the HO-1 effects on DC maturation was analysed.

2.2.4.3 Adenoviral HO-1 transduction of immature MDDC

In order to prove specificity for the observed effects on MDDC induced by the HO-1 inducer Co-PPIX, AdHO-1 transduction of immature MDDC was performed.

Adenovirus-based vectors have become gene delivery vehicles that are widely used for transduction of different cell types (T cells, DC), especially for quiescent cells, in basic research, gene therapy and in vaccine development.
Adenovirus is a non-enveloped, icosahedral virus of 60-90 nm in diameter with a linear, double-stranded DNA genome (30-40 kb). The capsid consists of 252 capsomers (Doefler et al, 1995). Following the attachment of adenovirus to host cell over the fiber protein and the cellular receptor CAR (Coxsackie- and adenovirus receptor), the virus uptake occurs by clathrin-dependent receptor-mediated endocytosis over RGD-motif, LTR and cell surface integrin molecules (Bergelson et al, 1997; Li et al, 2001; Tomko et al, 1997; Wickham et al, 1993).

Transduction assay

An Ad-HO-1 vector was used, which has been described by Otterbein (Otterbein et al, 1999). Ad-HO-1, AdGFP and Ad0 are without viral coding sequences and have low potential for adverse immune responses and toxicity. The vector cassette consists of a cytomegalovirus promoter.

Monocytes were negatively separated from human PBMC (see 2.2.4.1.2) and immature DCs were generated (see 2.2.4.2.1). Immature MDDCs were transduced in S2 laboratory either with Ad5-HO-1, Ad0 (Ad dl 312) (both kindly provided by Otterbein et al, 1999), or AdGFP in 48 well plate in 500 µL volume (Li E et al, 2001). Transduction was performed at a multiplicity of infection (MOI) of 1,000 in RPMI 1640 medium, supplemented with 2% (v/v) FCS and Penicillin/Streptomycin, over 3 hours. After the transduction cells were washed three times with PBS (without Ca^{2+} and Mg^{2+}) in sterile 1 mL Eppendorf tubes at 350 x g for 10 min at RT.
Additionally, 24 hours after transduction with AdGFP (gift by Dr. Michael Willem, Max-Planck-Institute for Neurobiology, Berlin) the transduction efficiency was determined by Flowcytometry (>70%).

Maturation of transduced immature MDDC

Transduced MDDCs were cultured with IL-4 and GM-CSF (10 ng/mL each) for additional 24 hours in VLE RPMI 160 medium (10% FCS and Penicillin/Streptomycin) in 6-well plates. Thereafter washed twice (350 x g for 10 min at RT) and MDDC maturation for AdHO-1 and Ad0 transduced cells was induced by further stimulation with 100 ng/mL LPS for 3 days. After the maturation, MDDCs were washed and i) lysed for analyse of HO-1 expression with classical real-time PCR (see 2.2.4.9.1), ii) labeled against CD86, CD83 and HLA-DR for surface analyse with flowcytometry (see 2.2.4.7, 2.2.4.7.5) and iii) applicated for MLR with 1 x 10^5 transduced cells and separated allogeneic CD4+ T cells (see 2.2.4.4.1.2).

2.2.4.4 Assays for antigen-presentation and T cell proliferation

2.2.4.4.1 Mixed leukocyte reaction (MLR)

The MLR is an in vitro method, which is based on T lymphocytes stimulation by with allogeneic cells (stimulator cells). There exists two kinds of MLR: one-way and two-ways MLR. In one-way MLR the allogeneic cells of the one donor are inhibited in proliferation by e.g. Mitomycin or γ-irradiation. Therefore selectively the proliferative T cell response of the other donor can be tested. In two-way MLR total T cell activity of both donors is tested. In general, T-helper cells show the strongest proliferation and cytokine synthesis in MLR. The T-helper cell proliferation can be determined by uptake of [^{3}H]-thymidine during the DNA synthesis or the decreased fluorescence of the membrane dye CFSE in the proliferated cells.

2.2.4.4.1.1 One-way MLR with human cells

For one-way MLR with human immune cells, the stimulator cells (PBMC or mature MDDC) were treated with Mitomycin (25 µg/mL 2 x 10^7 cells) over 25 min at 37°C in water bath and thereafter washed three times with VLE RPMI 1640 medium. Mitomycin inhibits cell proliferation by covalent binding to DNA. 1 x 10^5 stimulator cells/mL of were added to 1 x 10^6 responder cells/mL allogeneic (Th cells or PBMC) and cultured over 5 days in the presence or absence of Co-PPIX in 96-well round bottom plate in an end volume of 300 µL and run in triplicate. After five days 50 µL of supernatants were collected for IL-2 measurement (see 2.2.4.5.1, 2.2.4.5.3), and cell proliferation was quantified by incubation of cells with 25 µCi of [^{3}H]-thymidine during the last 6 hours.The cells were washed with Cell-Harvester in filter plates, dried for 20 min at 60°C in warm case, and 30 µL Scintillator was added. The DNA incorporated radioactivity was measured by a beta-counter.

2.2.4.4.1.2 One-way MLR with mouse spleen cells

At 24 hours after Intraperitoneal treatment with 20 mg/kg Co-PPIX or vehicle the mice (Balb/c and C57Bl/6) were killed and splenocytes were isolated (see 2.2.4.14). For MLR preparation, stimulator splenocytes (S) were incubated with mitomycin (25 µg/mL for 2 x 10^7) at 37°C for 25 min and washed three times with VLE RPMI 1640 medium. Stimulator cells (1 x 10^5 cells/mL) were mixed with allogeneic responder cells (R) (1 x 10^6 cells/mL) and cultured over 5 days in 96-well round bottom plates in a final volume of 300 µL. Assay run in triplicate. After five days cell proliferation was quantified by incubation with 25 µCi of [^{3}H]-thymidine during the last 6 hours. The cells were washed with Cell-Harvester in filter plates, dried for 20 min at 60°C in warm case, and 30 µL Scintillator was added. The DNA incorporated radioactivity was measured by a beta-counter.

Table 1. Combinations for allogeneic one-way MLR with splenocytes of Balb/c and C57Bl/6.

Balb/c	**x**	**C57Bl/6**	
R vehicle S vehicle		S vehicle R vehicle	**R-S-**
R Co-PPIX S vehicle		S vehicle R Co-PPIX	**R+S-**
R vehicle S Co-PPIX		S Co-PPIX R vehicle	**R-S+**
R Co-PPIX S Co-PPIX		S Co-PPIX R Co-PPIX	**R+S+**

2.2.4.4.1.3 Determination of T-helper cell proliferation with the membrane dye CFSE

CFDA-SE is a molecule, which diffuses passively through the cell membrane and does not show any fluorescence originally. Within the membrane the acetyl group of CFDA-SE will be cut off by cellular esterases, consequently the molecule CFSE remains in the membrane irreversibly. CFSE can be quantified by flowcytometry. It emits green fluorescence after activation by a 488 nm Argon laser. Since the membrane is "diluted" during cell proliferation to the daughter cells, membrane-associated fluorescence intensity of CFSE decreases to the half after each cell division.

CFDA-SE was dissolved in DMSO up to 5 mM stock solution, and for proliferation assay a 5 µM CFDA-SE in PBS was used. The CFDA-SE preparation and stain were performed according to the protocol of Lyons (Lyons et al, 1994).
For this purpose allogeneic human PBMCs were isolated, responder cells were treated with 5 µM CFDA-SE solution for 5 min at RT, and stimulator cells were incubated with mitomycin (25 µg/mL for 2 x 10^7 cells) over 25 min at 37°C in water bath. Unbound CFDA-SE and mitomycin were removed by three times washing with supplemented VLE RPMI 1640 medium.
Responder cells (1 x 10^6 cells/mL) were incubated with allogeneic stimulator cells (1 x 10^5 cells/mL) in 96-well round bottom plates for six days in the presence or absence of Co-PPIX at 37°C, 5.0% CO_2 and 95.0% rH. On day three, 50% of medium and Co-PPIX were carefully removed (without centrifugation) and replaced by new reagents. On day six the cells were resuspended, transferred into micronic tubes, washed with FACS buffer and labeled with CD4-PC5 antibody (see 2.2.4.7.5 in Monocytes).
The effects of Co-PPIX on proliferation of CFSE-labeled responder cells were measured by FACScan and analysed with CellQuestTM soft ware (Becton Dickinson, Heidelberg) in gated $CD4^+$ T cells.

2.2.4.4.2 Lymphocyte transformation test (LTT) in response to recall-antigen

LTT is based on the stimulation of memory T cell proliferation in response to a recall-antigen (e.g. tetanus toxoid) which is presented by the autologous antigen-presenting cells. LTT is qualified for determination of the successful immunization or for investigation of drugs effects on T cells or on APCs.

Either PBMCs (1 x 10^6 cells/mL) or MDDC (1 x 10^5 cells/mL) and autologous T-helper cells (1 x 10^6 cells/mL) were cultured with 0.08 IU/mL tetanus toxoid and Co-PPIX over 3 days in 96-well round bottom plate in a final volume of 300 µL. Assays run in triplicate. Cell proliferation was quantified by incubation of cells with 25 µCi of [^{3}H]-thymidine during the last 12 hours. The incorporated radioactivity was measured by a beta-counter (see 2.2.4.4.1.1).

2.2.4.5 Mediator detection in fluids

2.2.4.5.1 ELISA (Enzyme-linked immunosorbent assay)

ELISA is an immunological detection method for antigen determination in fluids, e.g. serum, plasma, homogenates, cell lysates and supernatants.

Principle of ELISA

The primary (mostly monoclonal) antibody is localized on a solid bottom of a 96 well microplate and the addition of a known antigen-standard concentration or an unknown test solution leads to a non-covalent antigen-antibody binding. After removal of unbound antigens by washing a secondary antibody is added, which is directed against free epitopes of the antigen. The secondary antibody can be directly or indirectly covalently labeled with an enzyme (indicator antibody), whose catalytic activity is initiated after application of the specific substrate (chromogen). The enzyme activity changes the substrate color and the addition of the stop solution (H_2SO_4) ends the catalytic activity.

Subsequently, the catalytic substrate turnover in the standard solutions is measured with a spectralphotometer. Thereafter a standard curve is calculated (e.g. by Soft-Max-Pro software - Molecular Devices) from which the unknown sample concentrations can be determined. In contrast to bioassays, ELISAs quantify antigen levels without differentiation regarding integrity and activity.
Alternatively to enzyme-linked immunoassays, the secondary antibody can also be conjugated with a fluorescence or with a radioactive molecule. Moreover, novel multiplex techniques allows the simultaneous measurement of several antigens in one sample (see Biosource Multiplex Bead-System).

Table 2. Design of the used cytokine and HO-1 ELISAs

	hIL-2	**hIL-10**	**hIL-12p70**	**hIL-12p40**	**IL-18**	**hTNF-α**	**HO-1**
Capture Ab	monoclonal	monoclonal	monoclonal	monoclonal	monoclonal	monoclonal	monoclonal
Detection Ab	polyclonal	monoclonal biotinylated	polyclonal	polyclonal	monoclonal	monoclonal	polyclonal
Substrate	Tetramethyl-bencidine + H_2O_2	Tetramethyl-bencidine + H_2O_2	NADPH	Tetramethyl-bencidine + H_2O_2	Tetramethyl-bencidine + H_2O_2	Tetramethyl-bencidine + H_2O_2	Tetramethyl-bencidine + H_2O_2
Enzyme Conjugate & Reaction	Horse radish Peroxidase Oxidation	Streptavidin-Peroxidase Conjugate Oxidation	Alkaline phos-phatase NADPH Dephosph. Reduction of Nitri-Blue-Tetracolimchloride	Horse radish Peroxidase Oxidation	Horse radish Peroxidase Oxidation	Horse radish Peroxidase Oxidation	Horse radish Peroxidase Oxidation
Emission Correction	450 nm λ 540 or 570	450 nm	490 nm λ 650 or 690	450 nm λ 540	450 nm	450 nm λ 490	450 nm
Standard	31.2-2000 pg/mL	7.8-500 pg/mL	0.625-40 pg/mL	31.2-2000 pg/mL	12.5- 1000 pg/mL	15-1500 pg/mL	0.78-25 ng/mL
Dilution	1:2 supernatants	1:5 supernatants	1:2 supernatants	1:2 supernatants	supernatants	1:10 super-natants	1:50 cell lysates

2.2.4.5.2 Multiplex bead-immunoassays

The principle of multiplex bead-immunoassays is based on cytokine-specific with fluorescence dyes labeled microspheres which are coated with the primary antibodies (Sandwich principle), indirectly fluorescence-labeled secondary antibodies and flowcytometry with two lasers. The used Luminex 100^{TM} system enables the simultaneous quantification of up to 100 analytes in one small sample (50 µL) in a 96-well-plate, whose bottom consist of 0.2 µm filter for washing of unbound proteins with using Vacuum-Millipore. The monoclonal antibodies are specific for a target protein or peptide and are covalently bound to 5,5 µm Polystyrene-Beads, which are internally dyed with differing ratios of two spectrally distinct fluorophores. Each fluorophore can have ten possible levels of fluorescent intensity, thereby allowing the usage of 100 spectra.

For each cytokine a different combination of fluorophores is defined. The secondary biotinylated detection antibodies are specific for free epitopes of the respective antigen. Addition of streptavidine-PE labels the bound antigens which can be determined in the Luminex system and analysed e.g. with Bio-Plex-Manager-Software (BioRad, München). The red diode classification laser (635 nm) illuminates the dyed beads and identifies the kind of target protein (e.g. IL-2 cytokine). The green reporter laser (532 nm) simultaneously excite phycoerythrin (PE), which is coupled to the detection antibodies, and the amount of orange fluorescence determines the quantity of the captured analyte.

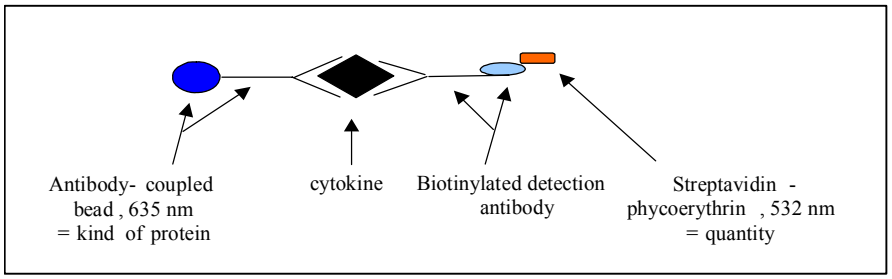


Figure 9. Principle of multiplex Bead-immunoassay
The detection of target protein occurred with antibody-coated fluorescence-labeled beads, which lead to identification of the kind of protein at 635 nm, and the biotinylated detection antibody / streptavidine-PE complex, which allows the determination of the analyte quantity.

The system was chosen for the simultaneous measurement of several cytokines in one sample. The samples were treated according to the protocol of the kit manufacturer's *Biosource.*

2.2.4.5.3 Cellular assays for cytokine secretion

Cytokine secretion in stimulated monocytes

Separated monocytes were incubated over 24 hours with different concentrations of Co-PPIX in presence of either 1 µg/mL LPS (for IL-10 TNF-α, IL-12p70) or 1 µg/mL LPS and 10 ng/mL IFN-γ (for IL-18) in 96-well plate in a final volume of 300 µL. Thereafter cells were centrifuged at 300 x g for 10 min at RT, and about 200 µL of supernatants were collected and stored at –80°C until cytokine measurement.

Cytokine secretion in LPS-stimulated MDDC

Immature MDDC were differentiated from monocytes (see 2.2.4.2.1), washed twice (350 x g, RT, 10 min) and stimulated in a concentration of 1 x 10^6 cells/mL with LPS (100 ng/mL) and Co-PPIX in 96-well plate in final volume of 300 µL for 24 hours. Thereafter cells were centrifuged (350 x g, RT, 10 min) and supernatants (200 µL) were stored in new 96-well plates at –80°C until determination of TNF-α, IL-12p40, IL-12p70 and IL10 levels.

Cytokine secretion in One way-MLR with human cells

IL-2 was determined in supernatants of MLR after five day culture of allogeneic stimulator and responder PBMC or of MDDC and allogeneic T-helper cells with Co-PPIX in 96 round well plates (see 2.2.4.4.1.1).

2.2.4.5.4 IL-4 in mouse ear homogenates

IL-4 levels in ear homogenates of mice were determined as parameter for a Th2-driven T cell-dependent skin inflammation in TMA-induced contact hypersensitiviy model (see 2.2.2.4.2). For this a self-made murine IL-4 ELISA was used.

Content of the self-made murine IL-4 ELISA:

1. coating buffer:	PBS	
2. Assay buffer:	PBS	100 mL
	BSA (2%)	2g
	Trimerosal (0.01%)	10 mg
3. Wash buffer:	500 mM Tris pH7.0-7.5 (MG 121.14) pH with Fixanal pure	60.57 g/L
	1:10 dissolved up to 50 mM Tris + 0.2% Tween 20	2 g/L

Mouse IL-4 Minikit (Endogen):

1. a) coating Ab
   b) Biotinylated detecting Ab
   c) Standard (34.9 ng/mL IL-4)
2. Streptavidin-conjugate (500 U/mL in Bidest)
   1:10,000 saturate in Assay buffer (100 µL/well = 5mU)
3. Substrate: TMB
   Stop solution: 0.5 Mol/L H_2SO_4
   IL-4 measurement at 450 nm

2.2.4.5.5 Bilirubin detection

HO-1 catalyzes the cleavage of the heme ring to form ferrous iron, carbon monoxide, and biliverdin. Biliverdin is rapidly reduced by biliverdin reductase to bilirubin, which can be measured as indicator of the enzymatic active HO-1.

Principle of bilirubin measurement

For bilirubin measurement we used bilirubin standard, sodium nitrite solution (0.07 mol/L) and bilirubin reagent (0.1% Sulfanil acid and 50% DMSO in 0.2 mol/L HCl with stabilizer). Briefly, bilirubin reagent (20 mL) was mixed with sodium nitrite (667 µL), and 200 µL of this solution were incubated with 100 µL either of standard or sample (serum / supernatants) for 5 min in 96-well plate at 30°C. Finally, the concentration of developed azobilirubin was measured photometrically at 520 nm adsorption (Spectral photometer). The used bilirubin standard concentrations diluted in distilled water were 0.5, 0.25, 0.125, 0.062, 0.031, 0.016, 0.008 mg/dL bilirubin.

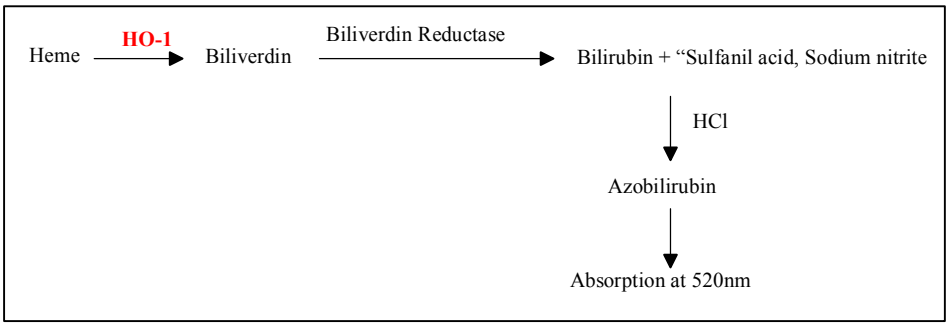


Figure 10. Bilirubin detection system
The bilirubin measurement bases on the reaction of total bilirubin with sulfanil acid and sodium nitrite in the presence of HCl whereby azobilirubin develops, which can be determined at 520 nm.

Bilirubin determination in mouse serum

For determination of bilirubin concentrations in mouse serum see 2.2.2.2.

Bilirubin determination in cell supernatants

Separated monocytes, T cells (MACS separation) and the monocytic THP-1 cells (1 x 10^6 cells/mL) were treated over 24 hours (monocytes, T cells) and over 24, 30 and 45 hours (THP-1 cells) with 20 and 50 µmol/L Co-PPIX in 48-well plate in an final volume of 500 µL. Thereafter cells were centrifuged (350 x g, 10 min, RT) and supernatants were collected for bilirubin determination.

2.2.4.6 HO-1 protein detection in cell lysates

Effect of Co-PPIX on HO-1 protein expression was investigated in monocytes, T cells and in MDDC. Cells (1 x 107 cells) were incubated over 6 hours with Co-PPIX in 10 mL plates and HO-1 protein expression in cell lysates was determined.

2.2.4.6.1 ELISA

Cells were lysed in extraction reagent (provided in HO-1 ELISA) and prepared for photometric HO-1 detection according to manufacturer's protocol (Stressgen) (see 2.2.4.5.1).

2.2.4.6.2 Western-Blot (WB)

Cell lysis

After Co-PPIX treatment cells were collected, washed twice with ice cold PBS (5 mL) and lysed in 500 µL fresh prepared lysis buffer for 10 min with agitate. All cell lysis steps were performed on ice, in ice cold tubes and in 4°C cold centrifuge. Thereafter lysed cells were transferred into a 1 mL Eppendorf-tube, vortexed twice for 30 sec, centrifuged at 4°C, 14000 rpm for 20 min, and the protein supernatants were transferred into 1 mL Eppendorf-tubes.

1 mL lysis buffer (4°C):

DTT (10 mM)	10 µL (= 0.1 mM)
Complete	100 µL (diluted with 1 mL distilled H_20)
Phosphatase-Inhibitor-Cocktail I	10 µL
phosphatase-Inhibitor-Cocktail II	10 µL
Extraction-Buffer	fill to 1000 µL

100 mL Basis-Extraction-Buffer (RT):

TRIS, pH 7,5	1.838 g (= 15 mM)
NaCl	0.7 g (= 120 mM)
KCl	0.19 g (25 mM)
EGTA	0.076 g (= 2 mM)
EDTA (Titriplex)	0.074 g (= 2 mM)
Triton X-100	0.5 mL (= 0.5%)

Measurement of protein concentration

In order to compare the protein expression in different conditions, total protein concentrations were measured with BCA Protein Assay Reagent kit and, before WB started, diluted to the same concentration.

Briefly, the test based on the Biuret reaction, which describes the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium. After addition of the bicinchoninic acid (BCA), which is responsible for the colorimetric detection and quantification of total protein, chelation of two molecules of BCA with one Cu^{1+} ion is formed and a water-soluble purple-colored product develops with absorbance at 562 nm. The method is not a true end-point method, because the final color continues to develop. The kit contains BCA reagent A (BCA), BCA reagent B (cupric sulfate) and albumin standard ampoules with range from 20-2000 µg/mL.

The protein quantification was performed according to manufacturer's protocol (Pierce) in 96-well plates with 25 µL sample volume. Protein measurement was done with Spectra Max 340 microplate reader (Molecular Devices, Heidelberg).

Electrophoresis

For electrophoresis, 30 µL sample mix (minimum 10 µg protein) was denatured for 5 min at 95°C in Eppendorf Thermomixer, cooled off on ice, mixed and then 20 µL were loaded to NuPAGE pre-cast gels (Bis-Tris-HCl buffered polyacrylamide Gel without SDS, pH 7) with 12 wells and acrylamide concentration of 4-12% (Invitrogen). See blue protein ladder (3 µL) was used, and the electrophoresis was run for 30-40 min at 200 V power with NuPAGE MES SDS buffer in Xcell II Mini-Cell. The expected molecular weight range for the protein ladder is from 200 to 3 kDa.

Sample mix (30 µL):

NuPAGE LDS-Sample-buffer (x 4)	7.5 µL
NuPAGE Reducing agent (0.5 M DTT)	2.5 µL
Sample	20 µL

NuPAGE MES SDS Running buffer (x 1):*

MES concentrate (x 20)	50 mL
H_20	950 mL

** 2-(N-morpholino) ethane sulfonic acid*

Into the upper (cathode) chamber (reduced proteins) of the electrophoresis equipment 200 mL MES (x1) + 500 µL of NuPAGE antioxidant were added. The NuPAGE Antioxidant prevent reduced proteins from reoxidizing during electrophoresis and this results in sharper and cleaner bands. For the outer buffer chamber 600 mL MES without antioxidant was used.

Protein transfer

After electrophoresis, the run proteins were transferred into pre-cut nitrocellulose membranes (soak in Transfer buffer) surrounded by filter papers and pads in transfer buffer for 1 hour at 30 V in Xcell II Blot Module in Xcell II Mini-Cell (Transfer buffer inside and H_2O outside of the transfer chamber). Thereafter nitrocellulose membrane was washed in TBST buffer and the protein transfer was controlled by Fast green stain.

1L NuPAGE Transfer-Buffer (1X) at RT:

Dest. H_2O	849 mL H_20
Transfer-buffer (20X)	50 mL
NuPAGE Antioxidant	1 mL
Ethanol	100 mL (=10%)

If transferring two gels within a blot unit:
200 mL Ethanol and 749 mL H_20

Reversible Fast green stain

For protein staining, nitrocellulose membrane was washed over 5 min with TBST buffer, 5 min stained in Fast green (0.1% in 1% CH_3COOH), washed 5 min in distilled H_20 and analysed (Epson scan and Adobe Photoshop). The stain was removed by 5 min incubation in 0.2 mol/L NaOH.

1 L TBST-T-buffer (x 10) at RT:

NaCl	87.66 g (= 150 mM)
TRIS, pH 7,5	30.29 g (= 25 mM)
Tween	0.5 mL/L (= 0.05%)
(Tween addition directly before usage)	

Western Blot (Detection of protein)

After removing of Fast green stain, membrane was washed over 5 min in TBST-T buffer, the unspecific bindings were blocked by 20 mL 5% BSE (in TBST-T) over 1 hour, and after washing (1 x 15 min and 2 x 5 min in TBST-T buffer) the membrane was incubated with the rabbit-HO-1 primary antibody (1:4000) in 20 mL of 0.5% BSE (in TBST-T) for 1 hour. The unbound primary antibody was removed by washing (1 x 15 min and 2 x 5 min in TBST-T buffer). Thereafter the anti-rabbit peroxidase-conjugated secondary antibody (1:4000) was added for 1 hour (20 mL of 0.5% BSE in TBST-T). After washing (1 x 15 min and 2 x 5 min in TBST-T buffer), the bound secondary Ab was detected with the chemoluminescent ECL-reagent (enhanced chemoluminescence) in a lightproof cassette with the exposition to the photosensitive Hyperfilm ECL. The film was developed with Curix 60 (Agfa). HO-1 protein was detected at 32 kDa by 5 min film exposition.

2.2.4.7 Flowcytometric assays

Background

Flowcytometry is a method for analysis of fluorescence labeled cells, which pass singly and successively in a liquid stream a reading point, and, therefore, each cell will be analysed individually.
The laser hits the cuvette flow chamber – each cell passes this point and the electronic detectors measure the forward (FSC) and sideward (SSC) scatters and the intensity of the fluorescence emission of each cell or particle. Light with determined wave-lengths is filtered by several dichroic filters and measured by corresponding detectors. The forward scatter characterizes the size and the sideward scatter the granularity of cells. The fluorescence intensity correlates with the light emission of the fluorochrom and with the density of an antibody-labeled antigen or other parameters quantified by fluorescence emission. Each cell is therefore characterized by several parameters.

Fluorescent molecules absorb light at one wavelength and emit light with a longer wavelength. In this process electrons of a molecule spring from a low energetic level to a higher one, which is energetic unstable and the transition into the stabile ground level leads to emission of a part of the energy as a long wavelength fluorescence (lower energy).
In flowcytometry the typically and frequently used fluorescence dyes are fluoresceinisothiocyanat (FITC) and phycoerythrin (PE). Both are excitable with an argon laser at 488 nm and emit light at different wavelengths. FITC emits light from 470-600 nm and PE from 540-620 nm. The overlap of the emission spectra have to be compensated by subtraction. With different fluorescence dyes and several lasers a cell analysis with more than eight fluorescences is possible.

Cells can be labeled with e.g. primary antibodies, secondary antibodies, Streptavidin-PE that binds to biotinylated antibody, or with fluorescence molecules, which intercale with DNA and RNA (e.g. propidium iodide). Cell functions can be also analysed by cytofluorometry. Examples are enzymatic conversions of fluorochromes or changes in ion concentrations.
In our studies FACScan instrument with a 488 nm argon laser was used. Data analysis war performed by CellQuest™ Software (Becton Dickinson, Heidelberg). Cells were either labeled with antibodies for immunophenotypic characterization, or with membrane dye for investigation of cell proliferation or cell functions were investigated by phagocytosis of fluorescence-labeled bacteria or by the conversion of a fluorescence dye by oxygen radicals in respiratory burst measurement.

General protocol for immunophenotypic characterization

Hundred microliters of 1 x 10^6 cells/mL (= 1 x 10^5 cells) were transferred into a micronic tube and washed with 750 µL FACS buffer at 4°C, 350 x g over 5 min. The supernatants were removed up to 100 µL solution, cells were resuspended, 5 µL of the respective fluorescence-labeled antibody was added (5 µL antibodies per 100 µL or 3 µL per 50 µL cell suspension) and, after vortexing an incubation was done for 30 min at 4°C in the dark. Thereafter labeled cells were washed in 750 µL FACS buffer at 4°C, 350 x g over 5 min, the supernatants were removed up to 100 µL, cells were resuspended and consequently measured.
Note, that intensity of fluorescence molecules decreased in light and high temperature, therefore labeled cells shall be stored in dark at 4°C until measurement.

FACS buffer:

500 mL PBS w/o Ca^{2+} and Mg^{2+}
+ 2% heat inactivated FCS
0,1% NaN_3

2.2.4.7.1 Purity of separated immune cell populations

The purity of separated monocytes and T cells was evaluated by flowcytometry using the following fluorescence-labeled monoclonal antibodies: anti-CD14 FITC (monocytes), anti-CD16 PE (NK cells), anti-CD19 PerCP (B cells) and anti-CD3 FITC (total T cells), anti-CD4 PE (T-helper cells), anti-CD8 PerCP (cytotoxic T cells). The minimal purity of MACS-separated cell populations was 90% for monocytes and 95% for $CD4^+$ positive cells.

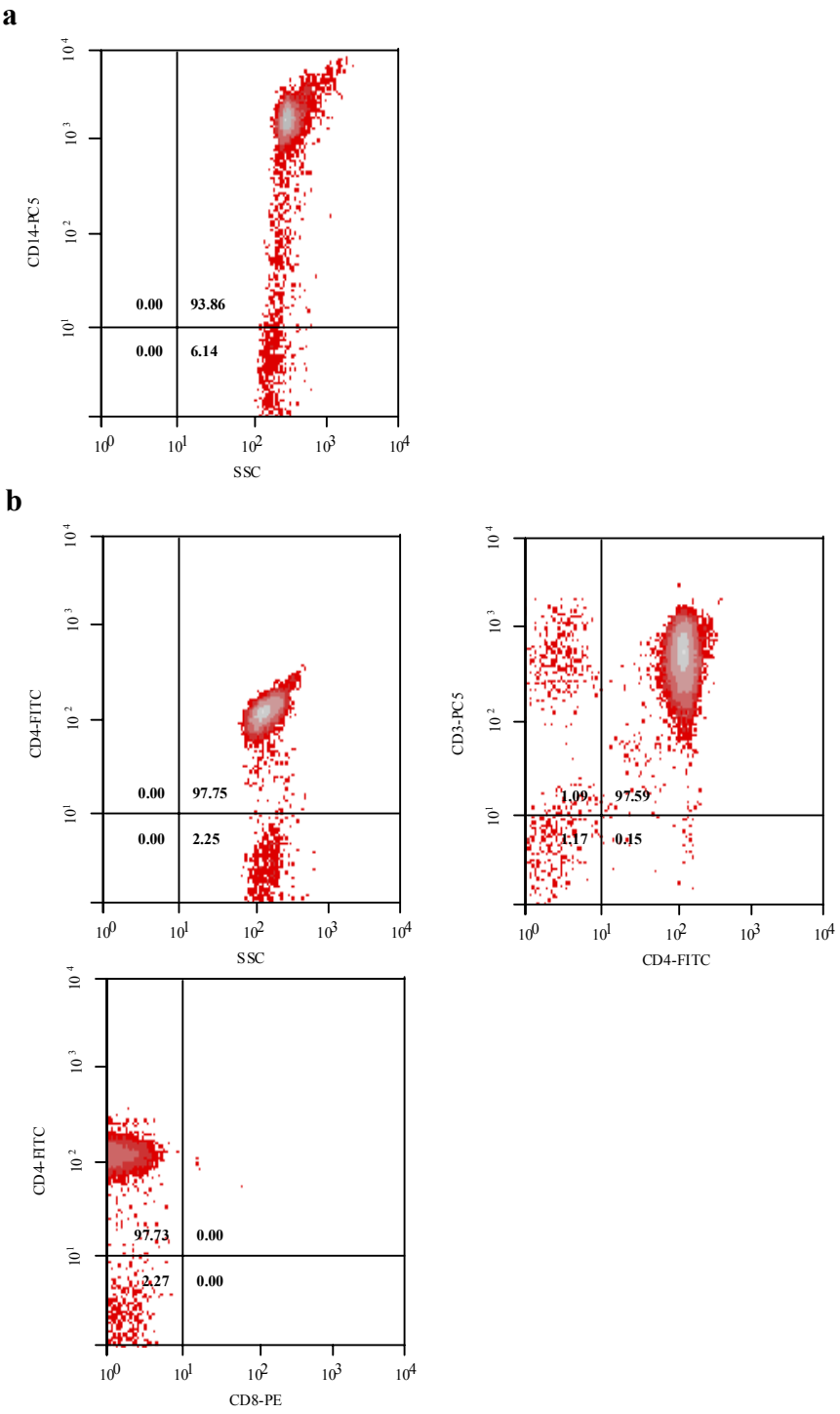


Figure 11. Purity of monocytes and b) Purity of CD4$^+$ positive cells
a) A representative purity analysis for monocytes is shown. Separated monocytes were labeled with anti-CD14, -CD16, -CD19, -CD3, -CD4 and -CD8 antibodies. All cells were gated for living cells. A dot plot of SSC (granularity) versus CD14-associated fluorescence is shown.
b) A representative purity analysis for CD4$^+$ positive T cells is shown. Separated cells were labeled with anti-CD14, -CD16, -CD19, -CD3, -CD4 and -CD8 antibodies. Cells were gated for living cells, and are shown in dot plots

2.2.4.7.2 Apoptosis and necrosis

Apoptosis describes programmed cell death, which occurs in many biological processes (homeostasis, disease, normal development) but also under toxic influence by drugs (high concentrations). During apoptosis, cells lose their phospholipid membrane asymmetry (earliest feature) and expose phosphatidylserine (PS) at the cell surface but their membrane integrity is intact. This process can be monitored by Annexin V-FITC labeling which is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein with high affinity for PS. Necrotic cells expose also PS at the outer membrane but, in contrast to early apoptotic cells, their membrane integrity is lost, whereby propidium iodide (PI) can intercale with the DNA. Consequently necrotic cells (PI positive) can be distinguished from solely Annexin V-FITC positive apoptotic cells.

Co-PPIX toxicity in human PBMCs

Pro-apoptotic and cytotoxic activity of Co-PPIX was determined with Annexin V-FITC apoptosis detection kit I in Co-PPIX treated cells. The kit consists of Annexin V-FITC, binding buffer and propidium iodide (PI). Cells were prepared for flowcytometric analysis according to manufacturer's protocol (BD Biosciences).
Briefly, PBMCs (1 x 10^6 cells/mL) were treated with Co-PPIX over 4 and 24 hours, 1 x 10^5 cells/mL (100 µL) were transferred into micronic tubes, Annexin V-FITC and PI were added and incubation in the dark at RT was done for 15 min. The proportions of apoptotic and necrotic cells were detected with FACScan flowcytometer in fluorescence 1 (Annexin) and 2 (PI).

2.2.4.7.3 Oxidative burst and phagocytosis

Phagocytosis and respiratory burst activities of monocytes and polymorphonuclear neutrophils constitute an essential arm of host defense against bacterial or fungal infections. The phagocytosis and oxidative burst tests allow the quantitative determination of leukocyte phagocytosis and burst in heparinized whole blood (not EDTA or citric acid!) by flowcytometry. Both tests were used in order to evaluate the effects of induced HO-1 on both processes in monocytes and granulocytes.

Oxidative burst in response to E. coli

In burst test, whole blood samples were stimulated with a physiological stimulant for phagocytes, unlabeled opsonized *E. coli*, in ratio of 25 bacteria to 1 white blood cell (2 x 10^7 *E. coli* in 20 µL to 8 x 10^5 leukocytes in 100 µL whole blood). After 10 min stimulation, the detection of oxidative burst was done by addition of the substrate dihydrorhodamine 123 (DHR), which is oxidized into fluorogenic rhodamine 123 (R123) by produced reactive oxidants by phagocytes after *E. coli* exposition.

After stopping of the burst activity (Lysing solution), DNA staining solution (Pi) is added and the oxidative burst is analysed by flowcytometry.

Because bacteria have partly similar scatter light properties as leukocytes, they can be excluded by DNA intercaled Pi by gating of human diploid cells. In the strongly Pi-labeled diploid cell population granulocytes and monocytes are gated according to size and granularity characteristics (forward and side scatter) and are analysed for R123 in fluorescence 1. All steps were performed according to the manufacturer's protocol (Orpegen Pharma).

Phagocytosis of FITC-labeled E. coli

In phagocytosis test, which described the internalization of alive or dead materials, FITC–labeled opsonized *E. coli* bacteria were used as phagocytosis indicator. After 10 min incubation and stopping of the reaction, Quenching solution was added for quenching the fluorescence of surface-bound FITC-labeled bacteria leaving the fluorescence of internalized particles unaltered. Thereafter erythrocytes were lysed and DNA staining with Pi was performed similar to the burst test. In the Pi-labeled diploid cells granulocytes and monocytes are gated according to light scatter characteristics (site and granularity) and analysed for internalized FITC-labeled bacteria in fluorescence 1. All steps were performed according to the manufacturer's protocol (Orpegen Pharma).

For both, the phagocytosis and burst experiments, whole blood was preincubated over 18 hours with different concentrations of Co-PPIX in 48 well plate in a final volume of 500 µL, and subsequently the stimulation with *E. coli* were started and analysed by flowcytometry.

2.2.4.7.4 CFSE labeling for cell proliferation measurement

CFDA-SE is a molecule, which diffuses passively through the cell membrane and does not show any fluorescence originally. Within the membrane the acetyl group of CFDA-SE will be cut off by cellular esterases, consequently the molecule CFSE remains in the membrane irreversibly. CFSE can be quantified by flowcytometry. It emits green fluorescence after activation by a 488 nm Argon laser. Since the membrane is "diluted" during cell proliferation to the daughter cells, membrane-associated fluorescence intensity of CFSE decreases to the half after each cell division.
For further details please see 2.2.4.4.1.3. For stain procedure see 2.2.4.7.5 in Monocytes.

2.2.4.7.5 Immunophenotypic analysis of monocytes and MDDC

Monocytes

Monocytes were isolated negatively (2.2.4.1.2) from human PBMCs and cultured over 24 hours with different concentrations of Co-PPIX in 48 well plate in a concentration of 1 x 10^6 cells/mL in an end volume of 500 µL. Thereafter HLA-DR and CD14 surface receptor expression were determined by flowcytometry. For this purpose hundred microliters of 1 x 10^6 cells/mL (= 1 x 10^5 cells) were transferred into a micronic tube and washed with 750 µL FACS buffer at 4°C, 350 x g over 5 min. The supernatants were removed up to 100 µL solution, cells were resuspended, 5 µL of the respective fluorescence-labeled antibody was added (5 µL antibodies per 100 µL or 3 µL per 50 µL cell suspension) and, after vortexing an incubation was done for 30 min at 4°C in the dark. Thereafter labeled cells were washed in 750 µL FACS buffer at 4°C, 350 x g over 5 min, the supernatants were removed up to 100 µL, cells were resuspended and consequently measured.

Monocyte-derived dendritic cells (MDDC) and macrophages

MDDC (iMDDC, mMDDC) were generated (see 2.2.4.2.1, 2.2.4.2.2), cultured with different concentrations of Co-PPIX and 100 µL (1 x 10^5 cells) were transferred into micronic tubes and labeled with monoclonal antibodies against CD14-FITC, HLA-DR-PE, CD86-PE, CD1a-PE and CD83-FITC. The effect of Co-PPIX on immunophenotypic DC differentiation and maturation was measured with FACScan and analysed with CellQuest™ software (Becton Dickinson). For stain procedure see above in Monocytes.

2.2.4.7.6 Adenoviral GFP transduction of immature MDDC and immunophenotypic analysis

24 hours after transduction with AdGFP the transduction efficiency was determined by Flowcytometry. About 1 x 10^4-1 x 10^5 cells were transferred into a micronic tube and washed with 750 µL FACS buffer at 4°C, 350 x g over 5 min. The supernatants were removed up to 100 µL solution, cells were resuspended and the intracellular GFP was analysed at fluorescence 1 (Fl 1).

After the maturation, with Ad0 and AdHO-1 transduced MDDCs were washed and labeled against CD86, CD83 and HLA-DR for surface analyse with flowcytometry. For stain procedure see 2.2.4.7.5 in Monocytes.

2.2.4.8 Histology and immunohistochemistry for mouse DNFB model

Mice were killed at 24 hours after DNFB challenge (see 2.2.2.4.1). The complete ears were removed and frozen in tissue freezing medium (Jung) over liquid nitrogen steam. Eight micrometer frozen serial sections (Cryostat) on object slide (Superfrost plus, Menzel) were dried (2-24 hours), fixed for 2 min with ice-cold 100% acetone, dried for 2 hour at RT (storage at –20 possible), sections were surrounded with Dako-Pen, 3 x 2 min washed with PBS (pH 7.2) for removing of the tissue freezing medium, and treated for 5 min at room temperature with 0.3% Peroxidase block (H202 in water) to inactivate endogenous peroxidases (Dako Cytomation).

2.2.4.8.1 Hematoxylin-Eosin (HE) stain

In order to analyse the tissue structure and cell infiltration in untreated (control) and in DNFB-challenged ears in mouse DNFB-induced contact hypersensitivity model (see 2.2.2.4.1) Hematoxylin-Eosin (HE) stain was performed.
Briefly, cryosections from control and DNFB-challenged ears were stained with the alcohol soluble hematoxylin (1:2 dilution in drinking water) for 10 min in 10% Scot`s tape water. Thereafter staining in 1% Eosin was performed over 60 sec. After shortly wash up in distilled water the sections were covered with Faramount (watery medium) and cover slips and were analysed 24 hours later at 20-fold extension under Microscope (Zeiss Axioplan) with Meta Vue program (Visitron).

2.2.4.8.2 Immunohistology for HO-1 expression and cell infiltration

Ear sections were washed 3 x 2 min with PBS and subsequently incubated for 5 min at room temperature with control serum of the secondary antibody host. After flow down (not washing), the diluted (PBS) primary antibody were added for 30 min, washed for 3 x 2 min with PBS and thereafter occurred the incubation with the biotinylated secondary antibody (15 min). After washing (3 x 2 min with PBS) Streptavidin-HRP (Pharmingen) was added (10 min), washed (3 x 2 min PBS) and the sections were stained by oxidation of the alcohol soluble AEC (3-amino-9-Ethylcarbacol) chromogenic substrate (1:200 in water). After 5 min incubation, three times rinsing with distilled water, nuclei were counterstained for 30-60 sec with the alcohol soluble hematoxylin (1:2 dilution in drinking water) and 10 min incubated in 10% Scot`s tape water (in drinking water) for precipitation of blue color. After rinsing with distilled water, sections were covered with Faramount (watery medium) and cover slip and analysed 24 hours later at 20-fold extension under Microscope (Zeiss Axioplan) with Meta Vue program (Visitron).

Table 3. Antibodies for immunohistological investigations

Primary Ab	Dilution	Secondary Ab	Dilution	Extension
HO-1 (rabbit) polyclonal Z1047	1:200	Goat anti rabbit –biotin	1:200	20x
CD4 (rat) monoclonal H129.19	1:20	Goat anti rat-biotin	1:200	20x
CD8 (rat) monoclonal 53-6.7	1:50	Goat anti rat-biotin	1:200	20x
Neutrophile (rat) monoclonal 7/4	1:200	Goat anti rat-biotin	1:200	20x
I-A/I-E (rat) monoclonal M5/114.15.2	1:200	Goat anti rat-biotin	1:200	20x

2.2.4.9 Quantitative gene expression analysis by real-time RT-PCR (TaqMan)

Either the classical primers and probes or Assays-on-Demand™ gene expression products (Applied Biosystems) were used for quantitative gene expression analyses. For both different systems various cell lysis, RNA separation, cDNA synthesis and real-time PCR protocols have been established.

2.2.4.9.1 Classical method

RNA separation

Cell populations (1 x 10^6 cells) were lysed in 600 µL of Invisorb RNA Kit II lysis solution in a sterile 1 mL Eppendorf tube, incubated on ice and 30 µL Adsorbin (RNA Kit) for DNA binding was added. The sample was strongly vortexed and incubated for 5 min on ice. After centrifugation (1 min, 14000 rpm, 4°C) the supernatant was transferred in a new Eppendorf tube and the Adsorbin-DNA-Pellet was rejected. Following, addition of 500 µL DEPC-water saturated phenol (Roth), 50 µL buffer A (Invisorb RNA Kit II), 100 µL chloroform and thereafter 15 sec. vortexing and holding on ice for 5 min. After centrifugation (10 min, 14,000 rpm, 4°C) RNA upper phase (up to 2 mm over the interphase) was transferred into a new tube, glycogen (2 µL) and isopropanol (the same volume as the upper phase) were applicated and RNA precipitated over 20-30 min at –20°C. After centrifugation (10 min, 14,000 rpm, 4°C), supernatant was rejected and 1 mL of 75% EtOH was added to the pellet and centrifuged at 14000 rpm, 4°C over 5 min. The last procedure was repeated, the pellet was dried at 60°C for 2 min and dissolved in 30 µL DNAse/RNAse free water. The obtained total RNA was quantified and qualified.

For gene expression analysis in tissues, skin or mouse organs were homogenized (IKA Ultra Turrax) in 800 µl lysis buffer (Applied Biosystems) and digested with 2 mg/mL Proteinase K for 1 hour. Thereafter 30 µL Adsorbin was added. For all further steps see above.

Quantity / Quality control of total RNA with capillary electrophoresis and fluorescence

The RNA quality / quantity was measured with Agilent 2100 Bioanalyzer and analysed automatically by Agilent 2100 Bioanalyzer Software, which calculates the ratio of 18s and 28s ribosomal peaks and reported the total RNA concentration in ng/µL related to ladder (RNA 6000 Ladder 150 ng/µL). For this reason RNA chips, Chip Priming station and RNA 6000 Nano Assay kit were used. The RNA 6000 Nano Assay kit consist of RNA Gel Matrix, RNA Dye concentrate, Spin Filters and RNA 6000 Nano Marker and they were treated according to the manufacture protocol. Briefly, filtered and dyed gel matrix were pipetted into defined well positions of the RNA chip and put on a chip priming station for distribution of the matrix in the microchannels of the chip.

The RNA marker, RNA 6000 ladder (150 ng/µL) and samples were dispensed into the wells and after vortex (IKA model MS2-S9) the prepared chip were placed in the Agilent 2100 Bioanalyzer, and the Eukaryote total RNA Nano assay protocol was run. Inside of the chip the total RNA moves through microchannels and is injected into the separation channel of the chip, were the RNA fragments are separated according to their size by means of molecular sieving. The laser excites RNA fragments and the fluorescence is detected at the detection point. Once detected the sample passes into the waste well. The quantitative range of total RNA is 25-500 ng/µL and the qualitative 5-500 ng/µL.

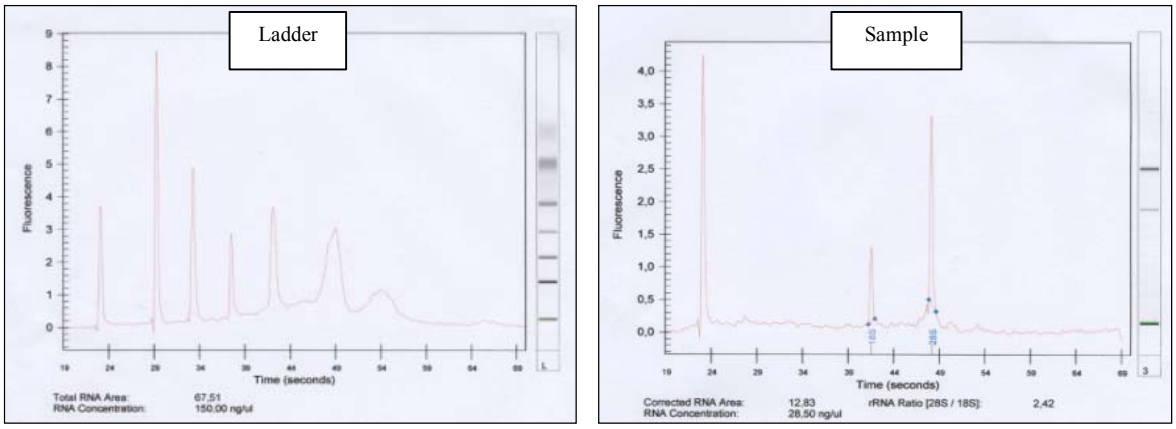


Figure 12. Quantity and quality control of total RNA
A typical example of RNA 6000 ladder (left) and total RNA in a sample (right). Major features of a successful ladder run are 6 RNA peaks and one marker peak (first at 24 sec.). Feature of a successful RNA sample run are one marker and two ribosomal peaks (18s, 28s), which ratio is calculated and reported in ng/µL related to ladder (150 ng/µL).

cDNA synthesis

cDNA is a DNA copy synthesized from mRNA. The used reverse transcriptase is a RNA-dependent DNA polymerase isolated from a retrovirus. As with other polymerases a short double-stranded sequence is needed at the 3´end of the mRNA which acts as a start point for the polymerase. This is pro-

vided by the poly(A) tail found at the 3´end of most eukaryotic mRNAs to which a short complementary synthetic oligonucleotide (oligo dT primer) is hybridized (polyT-polyA hybrid). Together with all four deoxynucleotide triphosphates, magnesium ions and at neutral pH, the reverse transcriptase synthesizes a complementary DNA on the mRNA template. Each mRNA molecule in the mixture with a poly(A) tail can be a template and will produce a cDNA in the form of a single stranded molecule bound to the mRNA (cDNA:mRNA hybrid).

In 0.5 µL Eppendorf tube 1 µg total RNA was fill up to max. 21 µL total volume with DNAse / RNAse free water, 2 µL OdT Primer were added and incubated at 75°C over 5 min. Thereafter the sample cooled off and was mixed with 8 µL 5x FSB buffer, 4 µL DTT, 4 µL dNTP mix, 2 µL RNAse inhibitor and 1 µL MMLV (Moloney Murine Leukemia Virus, 200 u/µL) for 10 min at RT. Thermal conditions for PCR were: 60 min at 42°C, 5 min at 95°C and 5 min at 4°C.

Real-time RT-PCR (TaqMan)

TaqMan indicates the probe used to detect specific sequences in Polymerase Chain Reaction (PCR) products by employing the 5´- 3´exonuclease activity of Taq-DNA polymerase. The TaqMan probe (20-30 bp), disabled from extension at the 3´end, consists of a site-specific sequence labeled with fluorescent reporter dye and a quencher dye. During PCR the TaqMan probe hybridizes to its complementary single strand DNA sequence within the PCR target. When amplification occurs the TaqMan probe is degraded due to the 5´- 3´exonuclease activity of Taq-DNA polymerase, thereby separating the quencher from the reporter during extension. Due to the release of the quenching effect on the reporter, the fluorescence intensity of the reporter dye increases.
During the entire amplification process this light emission increases exponentially, the final level being measured by spectrophotometry after termination of the PCR. Because increase of the fluorescence intensity of the reporter dye is only achieved when probe hybridization and amplification of the target sequence has occurred, the TaqMan assay offers a sensitive method to determine the presence or absence of specific sequences. Therefore, this technique is particularly useful in diagnostic applications (detection of pathogens and diseases). The TaqMan assay allows high sample throughput because no gel-electrophoresis is required for detection.

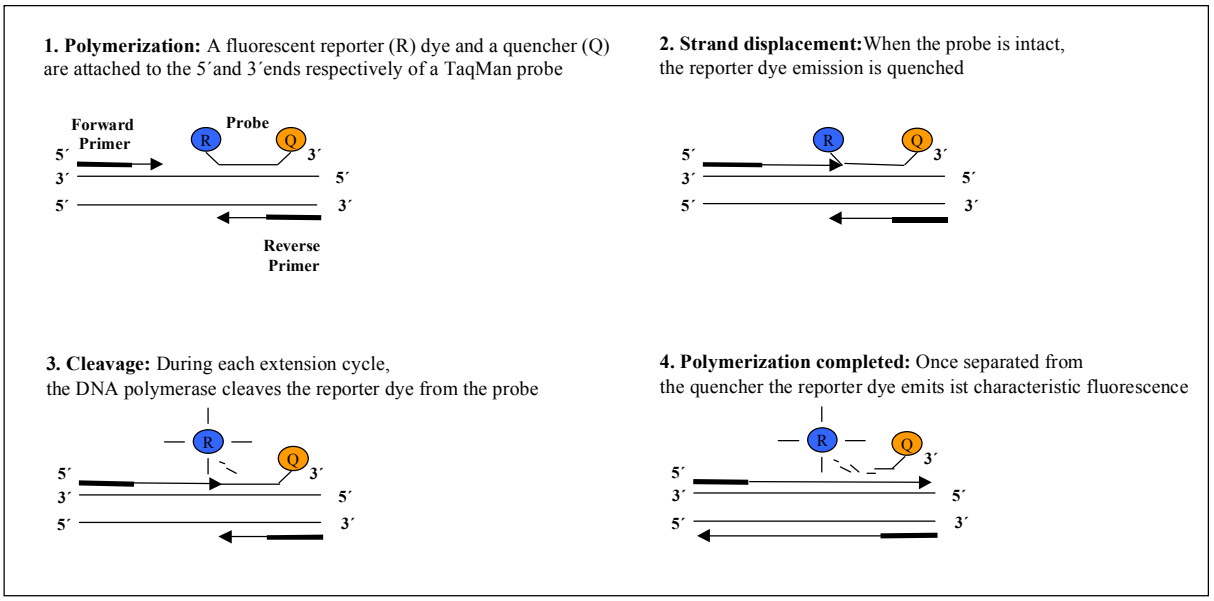


Figure 13. The principle of Real time PCR (TaqMan)
Real time PCR serves for the quantification of gene expression by successful polymerization (1-2) of primer and cleavage (3-4) of annealed probe on cDNA, which emits detectable fluorescence (R) after separation from quencher (4). Each detection of fluorescence means expression of wanted gene.

Real time PCR:

Preparation of 25 µL TaqMan sample in MicroAmp Optical Caps:
12.5 µL Master Mix
6 µL Primer
1 µL Probe
5.5 µL diluted cDNA (1 µL cDNA + 4.5 µL DNAse / RNAse free water)

Thermal conditions in each of the 40 cycles were: 15 s at 95°C and 1 min at 60°C.

The ABI Prism 7900HT Sequence Detection System background

It is a high-throughput real-time PCR system that detects and quantities nucleic acid sequences. Continuous wavelength detection from 500-600 nm allows the use of multiple fluorophores in a single reaction. To induce fluorescence, the 7900HT system distributes light from an argon laser excitation source to all sample wells. A spectrograph and charge-coupled device (CCD) camera spectrally resolve and collect the fluorescence emission from each sample. To quantify the amount of target in unknown samples, the ABI 7900HT system measured the sample Ct, which is defined by the section between Threshold and the increase (log phase) of fluorescence (Reporter) of each detectable gene.

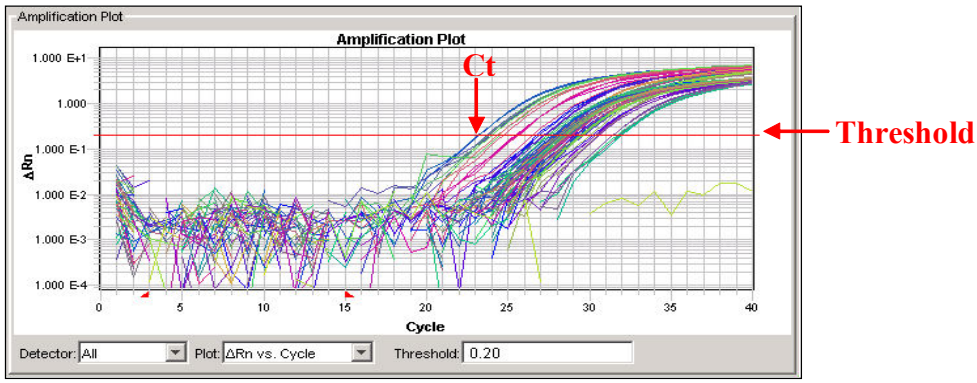


Figure 14. Amplification plot
Each line shows the course of cDNA amplification per well detected as reporter fluorescence. The course is described as number of cycle (X-axis) versus quantity of fluorescence (Y-axis). The threshold, positioned on the beginning of the logarithmic phase of the gene amplification in order to demarcate the gene rush (below), defines the exact cycle number for each gene detection (Ct).

Analysis of results

The mRNA expression data are given as fold expression of that of the house-keeping genes beta-actin (mouse organs) and hypoxanthine phosphoribosyltransferase (HPRT; human immune cells and skin).

2.2.4.9.2 Assays on Demand (AoDs)

Lyse of cells and RNA separation with ABI Prism 6100 Nucleic Acid PrepStation for Assays-on-Demand™

Cell populations were lysed in concentration of 1 x 10^6 cells/ 600 µL Cell Lysing Solution ½ diluted with PBS without Ca^{2+} and Mg^{2+}. The isolation of total RNA were performed according to manufacturer's protocol (Applied Biosystems) in ABI Prism® 6100 Nucleic Acid PrepStation with RNA Blood method Program in 96-well RNA-Purification Trays. The purification tray consists of an application specific membrane optimized to isolate precipitated RNA from the filtrates remaining after the elimination of genomic DNA. The chemistry includes two RNA Purification Wash solutions and a Nucleic Acid Purification Elution solution compatible with all downstream RT-PCR based applications (Assays-on-Demand™).

Briefly, the RNA purification trays inserted in PrepStation were pre-wet with wash solution 1 and 5 to 750 µL sample (Nucleic acid) per well were transferred. After several wash steps (Wash solution 1 and 2) under vacuum conditions serving for gDNA-removal the total RNA were eluated with Nucleic Acid Purification Elution Solution in a MicroAmp Optical-96-well-Reaction-Plate. The RNA quality were directly measured with Agilent Bioanalyzer.

RNA separation from whole blood with ABI Prism 6100 Nucleic Acid PrepStation

Venous whole blood was collected in Vacuette Tempus Blood RNA tube (Applied Biosystems), which contains a liquid RNA stabilization reagent enables a storage and stable transcript profile for whole blood RNA for at least 7 days at 4°C. For the RNA separation all used reagents and consumables were from Applied Biosystems and all preparation steps were made according to Applied Biosystem´s protocol.

Briefly, 3 mL blood was draw directly into Tempus Blood RNA Tube, shaked vigorously for 10-20 sec, diluted with 3 mL PBS and shaked again. After assembling the purification consumable (20 mL Reservoir, Large Volume RNA Prep Filter, Large Volume Adapter Plate) on the Waste Position of 6100 with Splash Guard, Filters were wetted with Wash Solution 1, diluted blood was loaded into 20 mL reservoir and washed with RNA Purification Wash Solution 1 and 2 after changing the reservoirs (5 mL reservoir). After several times washing until the filters are heme free (residual RNAse activity in heme!), filters were completely dried and incubated for 15 min with Absolute RNA Wash Solution for removing of gDNA.

After incubation for 5 min with Wash solution 2, filters were washed twice (RNA Purification Wash Solution 2) and the RNA was eluated into 2 mL microcentrifuge tube in 2 mL Tube Collection Plate on the Collection Position of 6100 after incubation over 2 min with Nucleic Acid Purification Elution Solution.

Total RNA quantity and quality:

See Quantity / Quality control of total RNA with capillary electrophoresis and fluorescence (2.2.4.9.1)

cDNA synthesis for RNA separated with ABI Prism 6100 Nucleic Acid PrepStation

Table 4. Components for cDNA synthesis

Component	*100 µL Reaction*
RNAse-free water	The volume of RNAse-free water will be 38.5-RNA sample in a 100 µL reaction
10x TaqMan RT Buffer	10.0
25 mM Magnesium Chloride	22.0
deoxyNTPs Mixture	20.0
Random Hexamers	5.0
RNAse Inhibitor	2.0
MultiScribe Reverse Transcriptase (50 U/µL)	2.5
Total	**61.5**

The used RNA concentration for cDNA synthesis was max. 1 µg/µL.

Random hexamers prime reverse transcription at multiple points along the transcript. For this reason, they are useful for either long mRNAs, or transcripts with significant secondary structure. First strand cDNA synthesis with random hexamers should be conducted at room temperature. Random hexamers are used for priming at non-specific points along the entire RNA template.

Table 5. Thermal Cycling parameters for cDNA synthesis in two-step PCR.

Step	Incubation	Reverse transcriptase	Reverse transcriptase Inactivation
	HOLD	HOLD	HOLD
Temperature	25°C	48°C	95°C
Time	10 min	30 min	5 min

Assays-on-Demand™

The Assays-on-Demand™ Gene Expression products are pre-designed primer and probe sets for quantitative real-time PCR gene expression studies. Assays-on-Demand™ consist of a 20x mix (feature 5´nuclease chemistry) of two unlabeled PCR primers and a Fam™ dye-labeled (5´end) TaqMan probe that incorporate MGB (minor groove binder at 3´end) technology and a non-fluorescent quencher at 3`end. The MGB is a small crescent-shaped molecule, which increases the melting temperature of probes by binding in the minor groove of a DNA duplex, allowing the use of shorter probes (13 bp) and consequently more binding stability and more differences between matched and mismatched probes. The non-fluorescent quencher (NFQ) virtually eliminates background fluorescence for better sensitivity and quantification precision. AoDs are designed to run under universal concentration conditions (900 nM primer/250 nM probe final concentrations).

Table 6. Components for Real Time PCR (TaqMan) using Assays-on-Demand

Reaction Component	Volume/Well (25 µL volume reaction)	Final Concentration
TaqMan Universal PCR Master Mix, No AmpErase UNG (2x)	12.5	1x
20x Assays-on-Demand Gene Expression Assay Mix	1.25	1x
cDNA diluted in RNAse-free water	11.25 (= 1 µL cDNA + 10.25 µL RNAse-free water)	
Total	**25**	

Table 7. Thermal Cycler Conditions for TaqMan with Assays-on-Demand

Thermal Cycler	**Times and Temperatures**			
	Initial setup		**Each of 40 Cycles**	
			Denature	Anneal/Extend
Sequence detection Systems 7900 HT	HOLD	HOLD	**Cycle**	
	UNG activation 2min 50°C	10 min 95°C	15 sec. 95°C	1 min 60°C

For Real-time RT-PCR (TaqMan), 7900HT Sequence Detection System and the analysis see 2.2.4.9.1.

Table 8. AoDs for gene expression analysis

AoDs	**Reference Sequence**
HPRT human	NM_000194
HPRT mice	NM_013556
HO-1 human	NM_002133
HO-1 mice	NM_010442
IL-10 human	NM 000572
IL-12p35 human	NM_000882
IL-12p40 human	NM_002187
TNF-α human	NM_000594

2.2.4.9.3 HO-1 mRNA expression analysis in human cells and tissue

2.2.4.9.3.1 Biomaterial from patients with skin inflammation

Skin from patients with psoriasis and nickel allergy

Paired lesional and non-lesional skin from psoriasis patients and challenged skin from donors with nickel allergy (1 cm^2, 0.3 mm thick, 10-30 mg) were homogenized. Total RNA and cDNA were generated with the classical method (2.2.4.9.1). For determination of HO-1 mRNA expression by real-time RT-PCR (TaqMan) AoDs for HO-1 and HPRT were used (2.2.4.9.2).

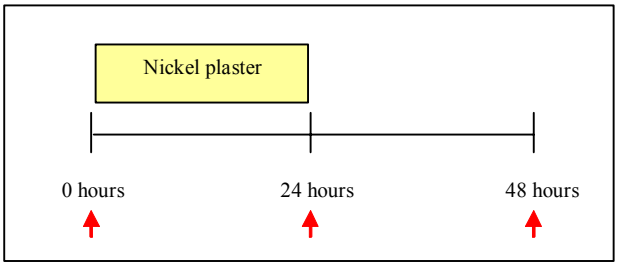


Figure 15. Schedule of nickel allergy challenge
Patients with known nickel hypersensitivity were treated over 24 hours with Nickel plaster and on day 0 (before), 1 and 2 challenged skin biopsies were taken for analysis of HO-1 mRNA expression.

Whole blood and separated peripheral blood immune cells from psoriasis patients

Whole blood was collected in Vacuette Tempus Blood RNA tube (Applied Biosystems), which contains a liquid RNA stabilization reagent. Monocytes and T cells were separated by anticoagulated whole blood samples by RosetteSep technique (see 2.2.4.1.3). RNA separation and cDNA synthesis was done according to the protocols for Gene expression analysis with AoDs for HPRT and HO-1 (see 2.2.4.9.2).

2.2.4.9.3.2 Co-PPIX treated or HO-1 transduced human immune cells

Human PBMC were treated with different concentrations of Co-PPIX over 2, 4, 6, 8 and 24 hours in 1 x 10^6 cells/mL in 6-well plates in a final volume of 5 mL. Monocytes, MDDC, $CD4^+$ und $CD8^+$ T cells (1 x 10^6 cells/mL) cells were incubated over 6 hours with Co-PPIX in 6 well plates in a final volume of 5 mL. Transduced iMDDC (Ad0, AdHO-1) were lysed after maturation for analyse of HO-1 expression with classical real-time PCR.
After centrifugation (300 x g, 10 min at RT) cells were lysed, RNA was isolated and cDNA was synthesized with the classical method. HO-1 and HPRT mRNA expression were determined with classical primers and probes (see 2.2.4.9.1).

2.2.4.9.4 Cytokine mRNA expression in MDDC

Immature MDDC were generated (see 2.2.4.2.1) and incubated in a concentration of 1 x 10^6 cells/mL over 6 hours with Co-PPIX and LPS (100 ng/mL) in 6-well plates in a final volume of 5 mL. Cells were centrifuged and lysed for gene expression with Assays on Demand (AoDs) (see 2.2.4.9.2). AoDs for human IL-12p35, IL-12p40, TNF-α, and IL-10 were used.

2.2.5 Statistical methods for ex vivo and in vitro assays

Results are presented as mean ± standard error of mean (SEM) or as mean ± standard deviation (SD). Statistical analyses for *in vitro* investigations in human immune cells were performed by Wilcoxon matched-pairs signed-ranks test and for *ex vivo* research in murine splenocytes by Mann Whitney U-test using SPSS software (SPSS Inc.). An error probability of $p<0.05$ was considered as significant.

3 Results

Heme oxygenase 1 (HO-1) can be induced by various forms of stress and drugs. It catalyses the cleavage of the heme ring into biliverdin, ferrous iron, and carbon monoxide. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase. For HO-1 induction and the degradation products of heme cytoprotection and antiinflammatory activity have been described (Figure 16).

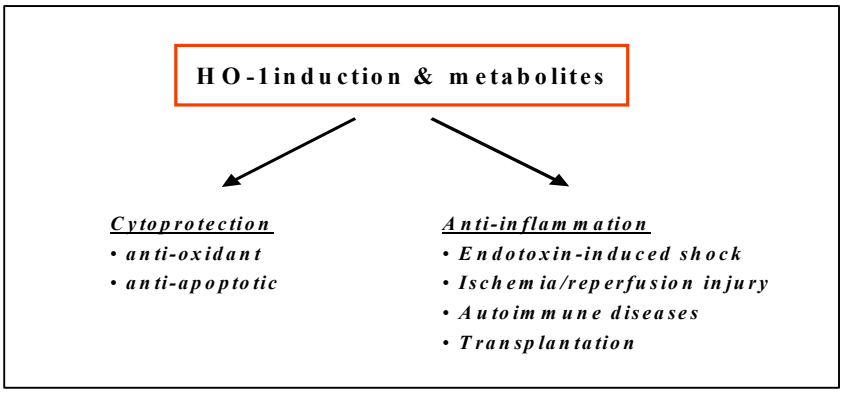


Figure 16. Cytoprotective and antiinflammatory effects of HO-1
For HO-1 induction and its metabolites cytoprotection with anti-oxidative and anti–apoptotic effects as well as antiinflammatory activity have been described in several animal models, e.g. in endotoxin-induced shock, ischemia/reperfusion injury, autoimmune diseases and in allotransplant models.

Because of that the question arose whether induction of HO-1 influences the T cell-dependent immune reaction. At first the HO-1 expression in skin inflammation and its physiological role have been investigated.

3.1 In vivo and ex vivo assays

3.1.1 Physiological increase of HO-1 expression in cutaneous inflammation

HO-1 is the rate-limiting enzyme in the degradation of heme. In addition to its role in iron metabolism, several findings indicate an important role of HO-1 in cellular protection against different kinds of stress and inflammatory damage. Therefore the HO-1 mRNA expression was determined in Th1 cell dependent skin inflammations, i.e. in psoriasis, human allergic contact dermatitis, and in acute dinitrofluorobenzene (DNFB)-induced contact hypersensitivity model in mice.

Psoriasis is a common chronic inflammatory skin disorder regarded as a type 1 T cell-associated disease with a prominent role for interferon (IFN)-γ and tumor necrosis factor (TNF)-α. The most obvious signs of the disease are hyperkeratosis and epidermal hyperplasia. The prevalence rate of psoriasis is approximately 2-3 % world-wide. Psoriatic lesions / inflammatory plaques are defined by above described strongly increased proinflammatory parameters, whose primary triggers are undefined so far (Bos et al, 2005; Schon et al, 2005;).
Nickel- and DNFB-induced contact eczemas are acute skin inflammations with delayed-type hypersensitivity reactions. The first contact with the allergen (nickel, DNFB) is without any skin-specific symptoms. The allergens are taken up by antigen-presenting cells (APC) of the skin which migrate into the regional lymph nodes where T cell recognition, activation and expansion take place. The second contact with the same allergen leads to a specific and fast immune response at the second contact location (independently from the first contact location). This response is mediated primarily by allergen-specific memory T cells of the Th1 type. The typical symptoms of contact dermatitis are edema (plasma extravasation) and swelling, redness and teleangiectasia, sometimes blisters, cell infiltration (granulocytes, monocytes, macrophages, lymphocytes) (Janeway et al, 2002).

3.1.1.1 HO-1 expression in psoriasis and allergic contact dermatitis in human

On the beginning of the HO-1 study the interest arose whether cutaneous inflammations are characterized by enhanced HO-1 expression. Therefore HO-1 mRNA expression was determined in lesional skin biopsy specimens of psoriatic patients and in paired non-lesional skin samples. An about 3-fold upregulation of HO-1 is found in lesional psoriatic skin in comparison to non-lesional skin (Fig.17a). These results are in accordance to a previous report describing enhanced HO-1 protein expression in lesional psoriatic skin (Hanselmann et al, 2001). Remarkably, in analyzed whole blood samples and in separated peripheral blood monocytes and T cells from psoriasis patients and healthy donors no differences in HO-1 expression were observed. Interestingly, the HO-1 mRNA expression values in healthy skin are always higher than in healthy whole blood.

The upregulation of HO-1 in psoriatic skin led to the questions whether HO-1 expression is also upraised in other skin inflammations and what is the kinetics of HO-1 gene expression. For this reason contact allergy reactions were triggered in probands with nickel allergy, and skin bioptates were taken off after different periods of time. Figure 17b shows that in acute nickel-induced allergic contact dermatitis in human an only negligible increase in HO-1 mRNA expression was observed in challenged skin after 24 hours in comparison to control (0 hours). Moreover, a 2.5-fold down-regulation of HO-1 expression was found at 48 hours after challenge. These data argue for a failing upregulation of HO-1 in allergic contact dermatitis or for a more accelerated kinetics in acute skin inflammation.

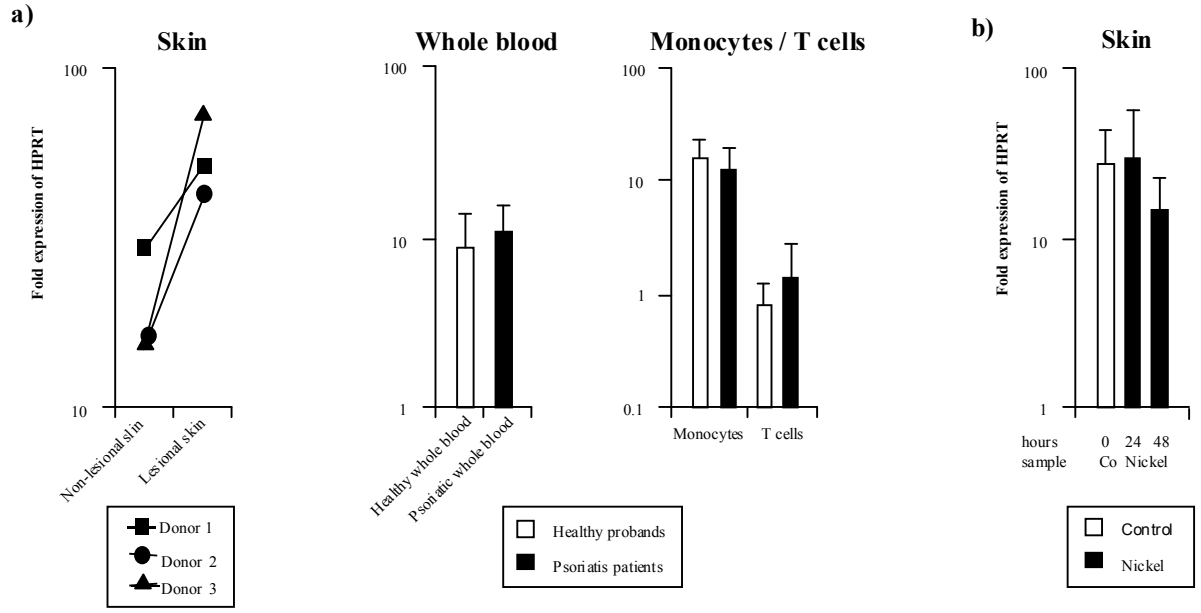


Figure 17. HO-1 mRNA expression in psoriasis and contact allergy patients
a) Paired HO-1 mRNA expression values in lesional and non-lesional skin from three psoriasis patients are given in logarithmic scale (left).
HO-1 mRNA expression in whole blood samples (22 healthy and 15 psoriatic donors, middle) and in separated monocytes and T cells (5 healthy and 10 psoriatic donors, right) are given as mean ± SD. Black bars represent data for psoriasis patients, white bars data for healthy blood donors.
b) HO-1 mRNA expression was determined in bioptates from untreated (Co, 0 hours) and in nickel-challenged skin of three donors with nickel allergy after 24 and 48 hours. Data are given in logarithmic scale as mean ± SD.

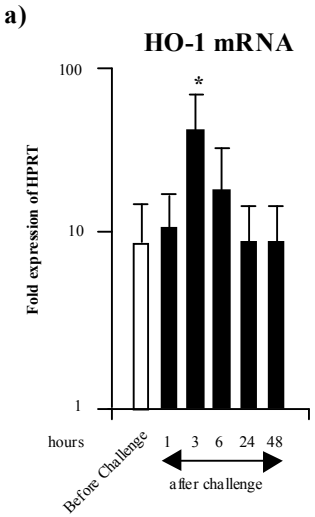
3.1.1.2 HO-1 is rapidly induced in an acute murine contact hypersensitivity model

In order to compare the cutaneous HO-1 mRNA expression in human and mouse skin inflammation and to define the precise early kinetics of cutaneous HO-1 mRNA expression in T cell-dependent skin inflammation, an acute DNFB-induced contact hypersensitivity (CHS) model in mice was performed. For this reason mice were sensitized with DNFB on two days and challenged 5 days later. Skin biopsy specimens from topically challenged flanks were taken before challenge (0 hour) and at 1, 3, 6, 24 and 48 hours after challenge, respectively.

Figure 18a shows the rapid and transient induction of HO-1 mRNA expression after DNFB challenge. A slight induction was observed at 1 hour after challenge, a significant 5-fold increase of HO-1 was found at 3 hours, and a slope down (2.5-fold higher than control) at 6 hours after challenge. At 24 hours after DNFB challenge the basal HO-1 values were reached again.

Topically challenged ears of DNFB-sensitized mice show typical inflammation symptoms defined by edema formation (plasma extravasation) and cell infiltration (granulocytes, neutrophils, macrophages, lymphocytes) and therefore increased ear weight and thickness. The cellular infiltration can be quantified by histology with Hematoxylin-Eosin (HE) (Figure 18b), which stains the nuclei and cell structure.

In DNFB-induced mouse ear inflammation model HO-1 protein expression and specific cell population markers in skin were determined by immunohistochemistry. Figure 18c shows the strongly increased HO-1 protein expression at 24 hours after DNFB challenge. At 24 hours after DNFB challenge (Fig. 18d) no $CD4^+$ T cells and only a slight infiltration of $CD8^+$ T cells (arrows) were detected in skin sections. There was, however, a strong expression of neutrophil-specific antigen and of MHC class II antigens (I-A/I-E) indicating a strong infiltration with granulocytes and macrophages, respectively.



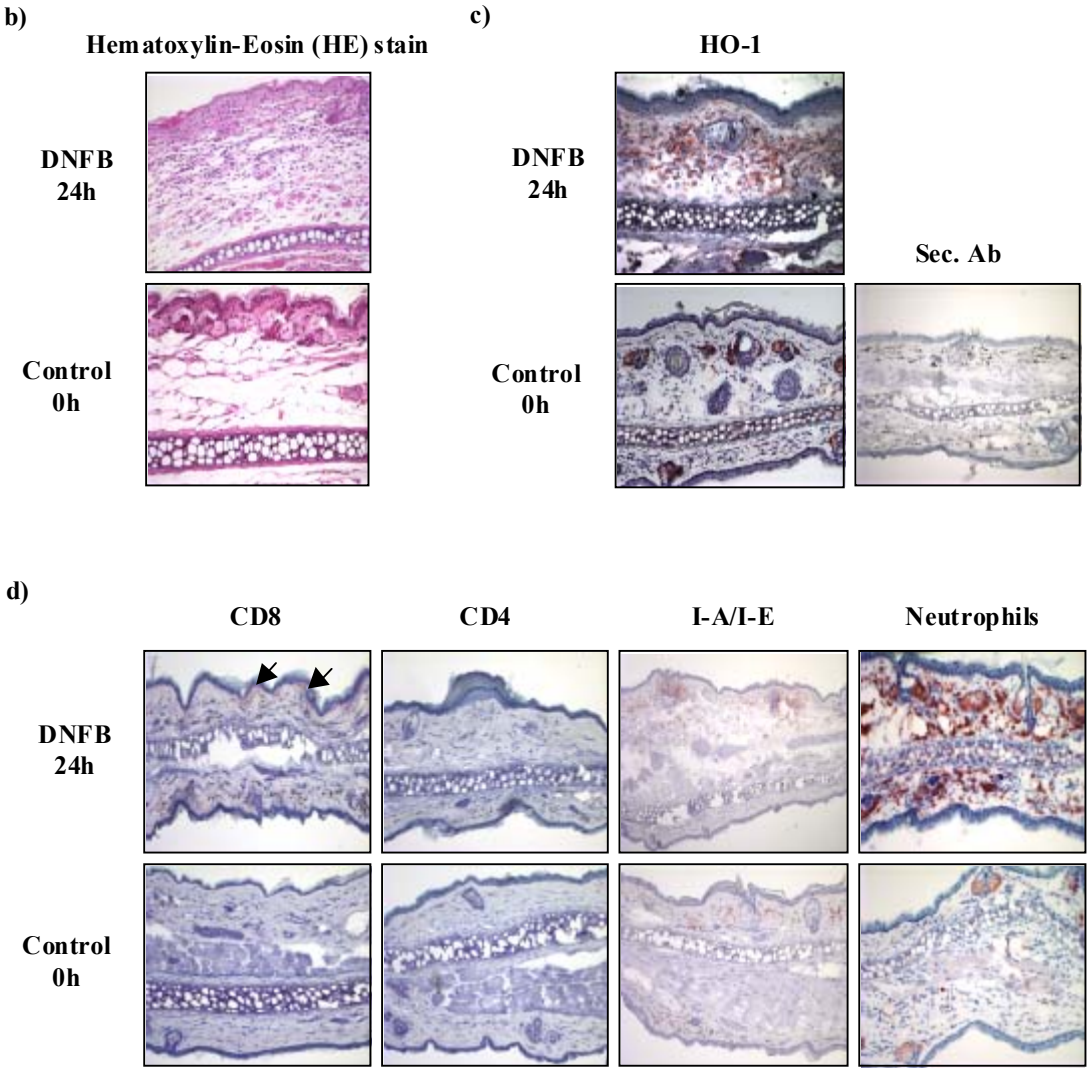


Figure 18. Cutaneous HO-1 expression and immune cell infiltration in acute DNFB-induced contact hypersensitivity in mice
a) HO-1 mRNA expression was determined in skin specimens of DNFB-challenged flanks of C57Bl/6 mice before and at 1, 3, 6, 24 and 48 hours after challenge. Mean ± SD values from five mice per time point are given in logarithmic scale.
$p<0.05$ versus sensitized mice, Mann-Whitney U test
b) Hematoxylin-Eosin stain of untreated and DNFB-challenged ears.
One representative example is shown at 20x enlargement.
c) Immunohistological detection of HO-1 in untreated and DNFB-challenged ears of sensitized NMRI mice at 24 hours after challenge.
Control for the non-binding of the secondary antibody is shown. One representative example is shown at 20x enlargement.
d) Immunohistological detection of CD8, CD4, I-A/I-E and neutrophils in skin of untreated (upper line) and DNFB-challenged ears of sensitized NMRI mice (lower line). For all proteins one representative example is given at 20x enlargement.

3.1.2 Inhibition of endogenous HO-1 activity increases experimental skin inflammation

To assess the physiological role of the HO-1 increase in skin inflammation the well-known inhibitor of HO-1 activity, Sn-PPIX, (Reeve et al, 1999) was used in DNFB-induced contact hypersensitivity model in mice. Sn-PPIX has been shown to block specifically the enzymatic domain of HO-1 whereby the oxidation of heme and the development of the antiinflammatory heme degradation by-products are suppressed (Blumenthal et al, 2005).

As shown in Figure 19 the application of the HO-1 inhibitor Sn-PPIX at 2 hours before DNFB-challenge dose-dependently increases the ear inflammation in DNFB-induced CHS model as determined by ear weight as parameter for ear edema formation.

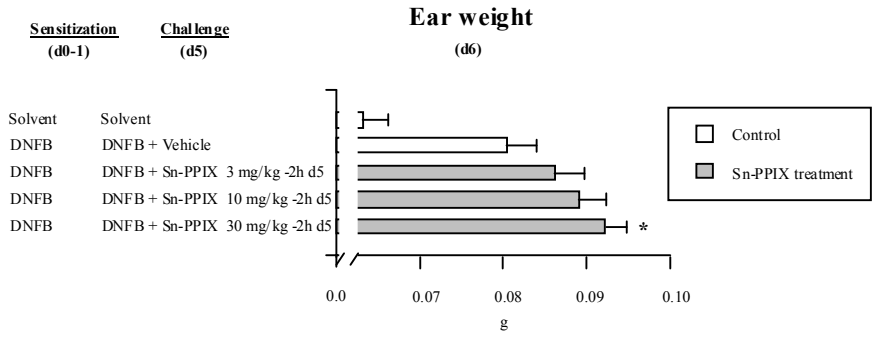


Figure 19. Treatment with Sn-PPIX, a selective inhibitor of HO-1 activity, augments skin inflammation in murine DNFB-induced contact hypersensitivity model
Intraperitoneal treatment with Sn-PPIX (3, 10 and 30 mg/kg) was performed at 2 hours before challenge in DNFB-induced CHS model in NMRI mice. Ear weights were determined at 24 hours after challenge. Data are given as mean ± SEM of 10 mice per group.
* $p<0.05$ vs. vehicle group, Fieller´s test

Taken together, the physiologically increased HO-1 activity can be suppressed by a specific HO-1 inhibitor resulting in increased skin inflammation. This indicates an important role of HO-1 for the limitation / resolution of inflammatory skin injury.

3.1.3 Pharmacological induction of HO-1 activity by Co-PPIX inhibits skin inflammation and depresses T cell immunity in mice

The physiological increase of HO-1 expression in cutaneous inflammation and the enhanced inflammatory skin injury caused by inhibition of HO-1 activity raised the question whether cutaneous inflammation can be inhibited / prevented by pharmacological (super)induction of HO-1. For this purpose the well-characterized inducer of HO-1 expression, Co-PPIX, was used. Co-PPIX has been shown to counteract inflammatory injury in several disease models (Blumenthal et al, 2005; Reeve et al, 1999). In our study Co-PPIX was applied for the first time for assessing the effect of HO-1 induction in experimental skin inflammation.

3.1.3.1 Co-PPIX treatment induces HO-1 mRNA expression and HO-1 activity in mice

At first, the Co-PPIX capacity for induction of HO-1 mRNA and enzymatic activity was investigated in mice. HO-1 mRNA expression was determined in organs of Balb/c mice at 6 hours after i.p. treatment with 15 mg/kg Co-PPIX. A strong HO-1 induction by Co-PPIX was observed in all investigated organs except the brain. Figure 20a shows the strongest HO-1 induction in lymph nodes, bowel, liver, and skin (32-fold).

For assessment of HO-1 activity, the heme-cleavage by-product bilirubin was measured in the sera of mice after treatment with Co-PPIX for 1, 2 or 3 days. The systemic bilirubin concentration increased in a time-dependent manner in comparison to vehicle treatment for 3 days (Fig. 20b) indicating that Co-PPIX is a potent inducer of functionally active HO-1 *in vivo*. Figure 20c shows a photo of mice sera which changed in color (bilirubin) dependent on the duration of Co-PPIX treatment.

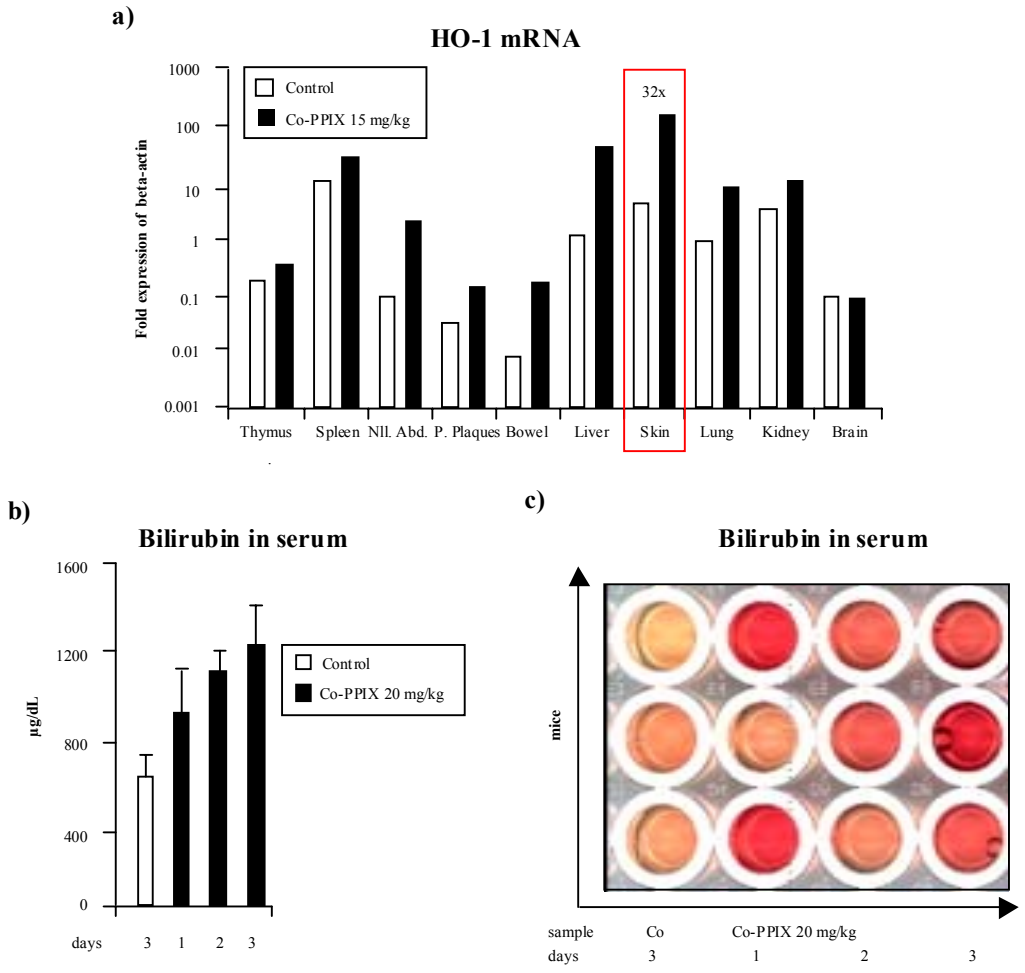


Figure 20. Co-PPIX treatment induces HO-1 expression and activity in mice.
a) HO-1 mRNA expression was determined in different organs of Balb/c mice at 6 hours after i.p. administration of 15 mg/kg Co-PPIX (black bars) or vehicle (white bars). Data from one mice are given in logarithmic scale.
b) Bilirubin concentrations were determined in sera of Balb/c mice at 1, 2 and 3 days after i.p. application of 20 mg/kg Co-PPIX in comparison to vehicle treatment for 3 days (Co). Data are presented as mean ± SEM of three mice per group.
c) The photography shows the color changes in sera of Balb/c mice at 1, 2 and 3 days after i.p. application of 20 mg/kg Co-PPIX in comparison to vehicle treatment for 3 days (Co).

3.1.3.2 HO-1 induction prevents skin inflammation in murine contact hypersensitivity models

To address the question whether pharmacological induction of HO-1 inhibits experimental skin inflammation, the specific HO-1 inducer Co-PPIX (Reeve et al, 1999) was used in dinitrofluorobenzene (DNFB)- and trimellitic anhydride (TMA)-induced murine contact hypersensitivity (CHS) models. These models are either characterized by a strong Th1 response (DNFB) or by a predominant Type 2 cytokine profile (TMA) (Dearman et al, 1996).

Treatment with the HO-1 inducer Co-PPIX led to a dose-dependent inhibition of ear inflammation in DNFB-induced CHS when applied at 6 hours before challenge. The antiinflammatory effect of Co-PPIX was specifically abolished by simultaneous treatment with the inhibitor of HO-1 activity, Sn-PPIX (Fig. 21a). Interestingly, Co-PPIX treatment during the sensitization phase with DNFB (day 0-1) caused also a significant reduction in ear edema formation (Fig. 21b) indicating that HO-1 affects the initiation of contact sensitivity.

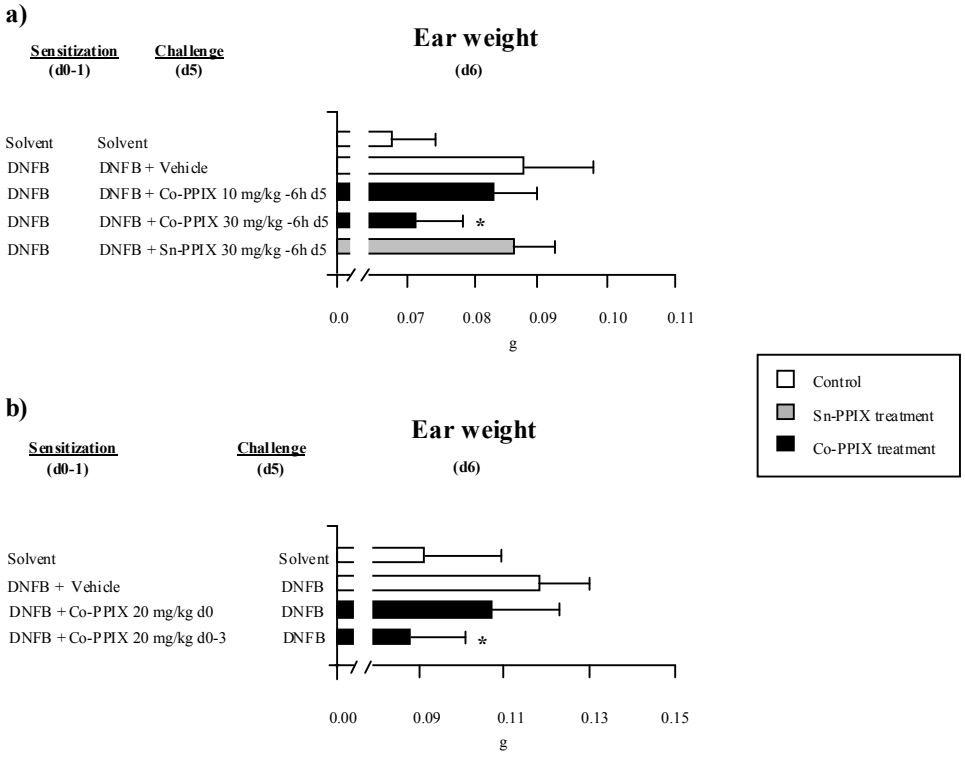


Figure 21. Co-PPIX inhibits inflammation in murine DNFB-induced contact hypersensitivity model, and the anti inflammatory effect is abolished by the HO-1 inhibitor Sn-PPIX
a) In DNFB-induced mouse CHS model i.p. treatment with Co-PPIX (30 mg/kg) was performed at 6 hours before challenge with DNFB either alone or in parallel with Sn-PPIX treatment (30 mg/kg). Ear weights were determined at 24 hours after challenge.
b) In DNFB-induced mouse CHS model i.p. treatment with Co-PPIX (30 mg/kg) was performed during the sensitization phase (day 0 + 1). Ear weights were determined at 24 hours after the DNFB challenge at day 6.
Data are given as mean ± SEM of 10 mice per group.
* $p<0.05$ vs. vehicle group, Fieller´s test

In TMA-induced CHS model, HO-1 induction by Co-PPIX treatment at 24 hours before challenge led to a dose-dependent decrease in edema formation (Fig. 22a) as well as to a suppression of IL-4 levels in ear homogenates (Fig. 22b).

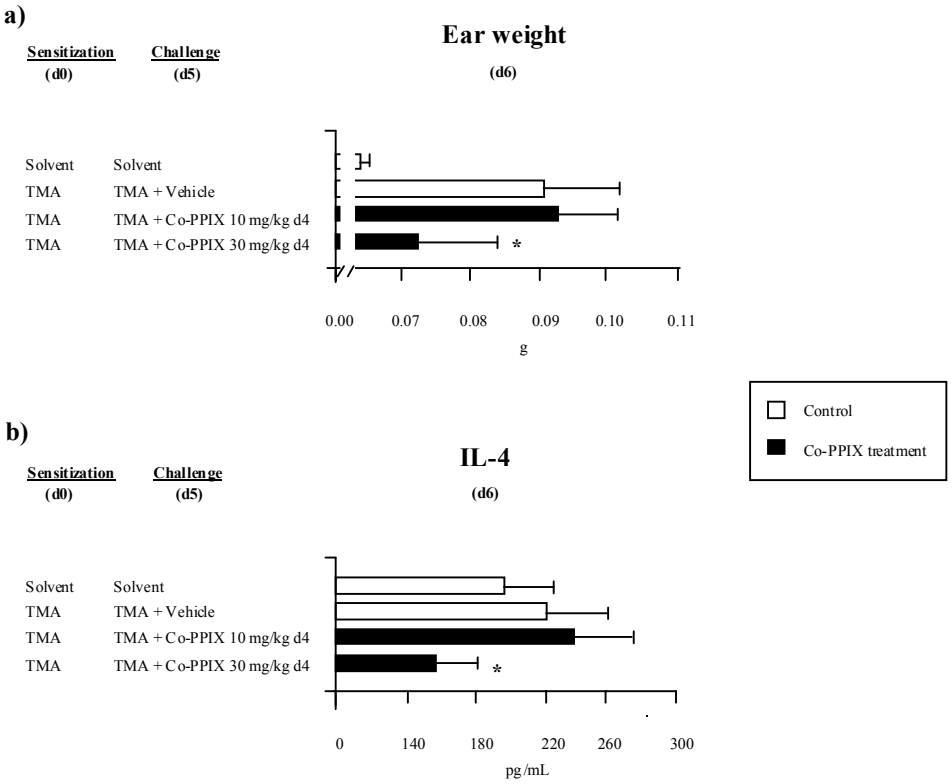


Figure 22. Co-PPIX inhibits inflammation in murine TMA-induced contact hypersensitivity model
a) In TMA-induced mouse CHS model i.p. treatment with Co-PPIX (30 mg/kg) was performed at 24 hours before the challenge at day 5. Ear weights were determined at 24 hours after challenge.
b) IL-4 concentrations in supernatants from ear homogenates at 24 hours after TMA challenge at day 5 were determined by ELISA.
Data are given as mean ± SEM of 10 mice per group.
* $p<0.05$ vs. vehicle group, Fieller´s test

Taken together, HO-1 induction by Co-PPIX suppresses the type 1 and type 2 T cell-dependent skin inflammation in mouse contact hypersensitivity models.

The inhibition of the T cell-dependent inflammation in mice CHS models led us to ask for the immunological mode of action of HO-1. Therefore the effects of HO-1 induction on T cell immunity were tested in an *ex vivo* assay.

3.1.3.3 HO-1 induction by Co-PPIX depresses T cell immunity in ex vivo mixed leukocyte reaction

To get evidence about the immunological mechanism of immunosuppressive effects of Co-PPIX, the alloreactive lymphoproliferative response of spleen cells from Co-PPIX treated mice was analyzed in mixed leukocyte reaction (MLR). Whereas some decrease of splenocyte proliferation in MLR was observed after Co-PPIX treatment of the donor mice for the responder splenocytes, very profound inhibition was demonstrated after treatment of donors for the (mitomycin-treated) stimulator cells (Fig. 23). Co-PPIX treatment of both, stimulator and responder splenocyte donors, did not further augment the inhibition. These data suggest a dominant effect of Co-PPIX *in vivo* treatment on antigen presenting cells.

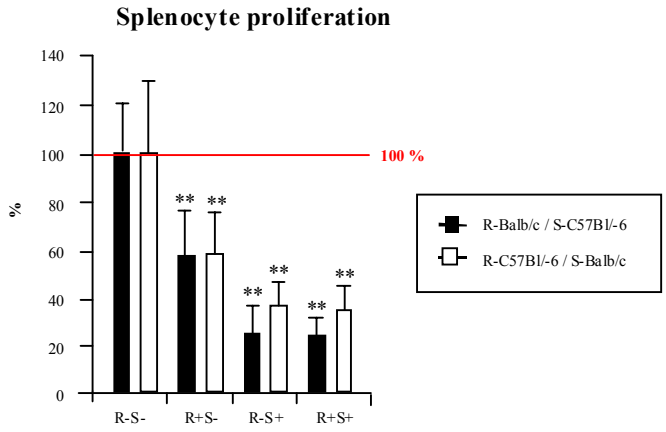


Figure 23. Co-PPIX treatment of mice inhibits alloreactive splenocyte proliferation in ex vivo culture
Allogeneic splenocytes were harvested from Balb/c and C57Bl/6 mice at 24 hours after i.p. treatment with vehicle or 20 mg/kg Co-PPIX and cultured in MLR using either responder cells (R) from Balb/c and (mitomycin-treated) stimulator cells (S) from C57Bl/6 mice or conversely. Co-PPIX treatment was performed either in responder (R+S-) or stimulator cell donors (R-S+) or in both (R+S+). Splenocyte proliferation data from 5 independent experiments are given as mean ± SEM of the percentages of the values in MLR using splenocytes from not Co-PPIX-treated donors (R-S-) (R-Balb/c 452,894 cpm; R-C57Bl/6 279,053 cpm).
** $p<0.01$ vs. the respective control without Co-PPIX treatment (R-S-), Mann-Whitney U test

3.2 In vitro assays

3.2.1 Effects of HO-1 induction in human PBMC

3.2.1.1 Co-PPIX treatment does not show cytotoxic effects

The importance of HO-1 induction for resolution/reduction of CHS inflammation and the inhibition of T cell proliferation in *ex vivo* experiments led to the investigation of HO-1 immunological mechanism in *in vitro* assays in human immune cells. Before the assays were started the Co-PPIX toxicity in monocytes and lymphocytes was investigated.

Apoptosis describes programmed cell death, which occurs in many biological processes and under toxic influence by drugs (e.g. at high concentrations) and can be measured as loss of the membrane asymmetry with exposition of phosphatidylserine (PS). In early apoptotic cells the membrane permeability is intact in contrast to necrotic cells, whereby DNA intercaled propidium iodide (Pi) distinguishes between apoptotic and necrotic cells.

In human PBMC treated over 4 and 24 hours with different concentrations of Co-PPIX the binding of Annexin V-FITC for PS (apoptosis) and the Pi-DNA intercalation (necrosis) were measured. For analyzed monocytes (Fig. 24a-d) and lymphocytes (Fig. 24e-h), Co-PPIX treatment results in an only negligible change in the percentage of apoptotic and necrotic cells in comparison to control cultures (maximum change about 2% of the whole cell population).

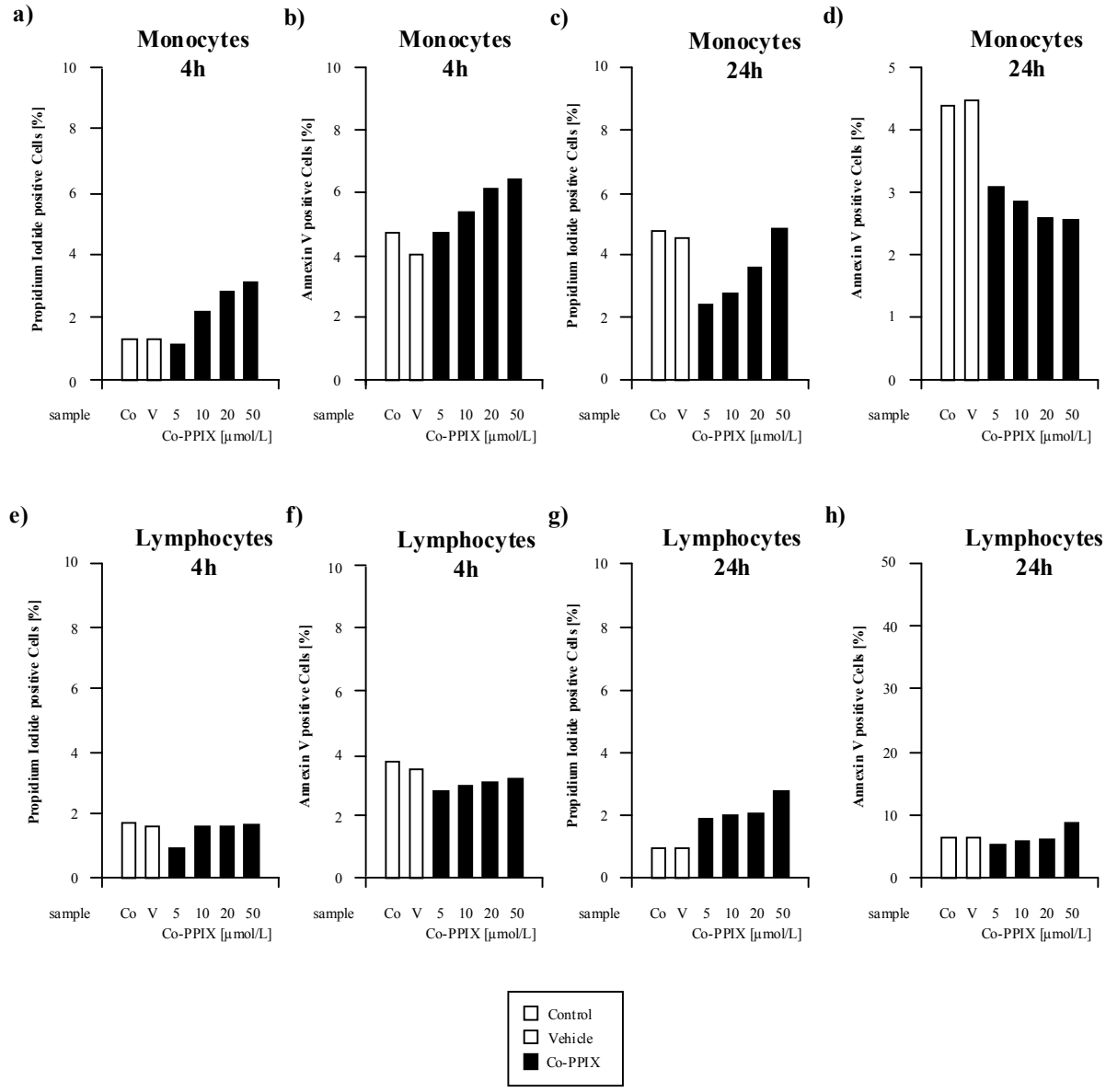


Figure 24. Co-PPIX as a strong inducer of HO-1 does not show toxicity in human monocytes and lymphocytes
Human PBMC were treated either with medium (first white bars, Co) or vehicle (second white bars, V) or with different concentrations of Co-PPIX (black bars) over 4 (a-b, e-f) and 24 hours (c-d, g-h). Co-PPIX effect on percentages of Pi (a, c, e, g) and Annexin-V (b, d, f, h) positive cells was determined in gated monocytes (a-d) and lymphocytes (e-h) from PBMC culture. Data from one representative experiment are given.

3.2.1.2 Co-PPIX induces HO-1 mRNA expression in PBMC

In order to find out the kinetics of HO-1 induction by Co-PPIX, human PBMC were incubated over 2, 4, 6, 8 and 24 hours, respectively, with different concentrations of Co-PPIX. As shown in Figure 25 the maximal HO-1 mRNA induction depends on Co-PPIX concentrations and the length of incubation. The lower Co-PPIX concentrations reach the HO-1 expression maximum later and the higher earlier. For all further investigations was decided to use a 6 hour incubation since no further HO-1 induction was observed by a longer incubation with an intermediate Co-PPIX concentration.

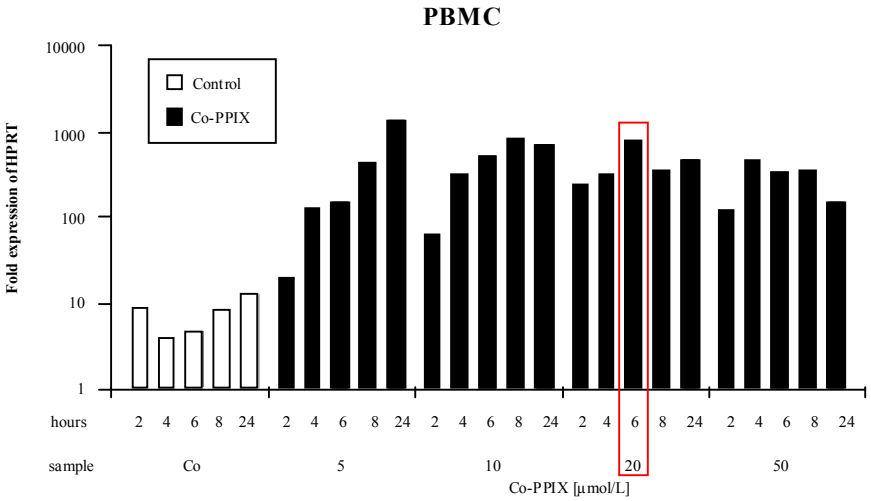


Figure 25. Kinetics of Co-PPIX-induced HO-1 mRNA expression in human PBMC
HO-1 mRNA expression in human PBMC was determined at 2, 4, 6, 8 and 24 hours after treatment with vehicle (white bars) or with 5, 10, 20 and 50 µmol/L Co-PPIX (black bars). Results from one experiment are given.

3.2.1.3 HO-1 induction depresses T cell immunity in vitro

In order to determine whether the finding of an inhibited alloreactive mouse splenocyte proliferation can be translated into the situation in human, mixed leukocyte reaction (MLR) with allogeneic human PBMC as well as lymphocyte transformation test (LTT) with human PBMC and the recall antigen tetanus toxoid were performed. In both assays Co-PPIX significantly and dose-dependently suppresses T cell proliferation (Fig. 26a). Additionally the secretion of the proliferation promoter IL-2 is inhibited by Co-PPIX (Fig. 26b).

Figure 26c shows the results of a flowcytometric proliferation assay with the membrane dye CFSE, whose intensity is diluted to the half after each cell division. A representative dot plot for proliferating CD4+ T cells is given (Fig. 26c, left). Percentages of proliferated $CD4^+$ T cells (loss of CFSE fluorescence) were analyzed using an appropriate marker (26c, right). The declining proportions of cells with diminished CFSE fluorescence demonstrate that the proliferation of $CD4^+$ T cells is dose-dependently inhibited by Co-PPIX.

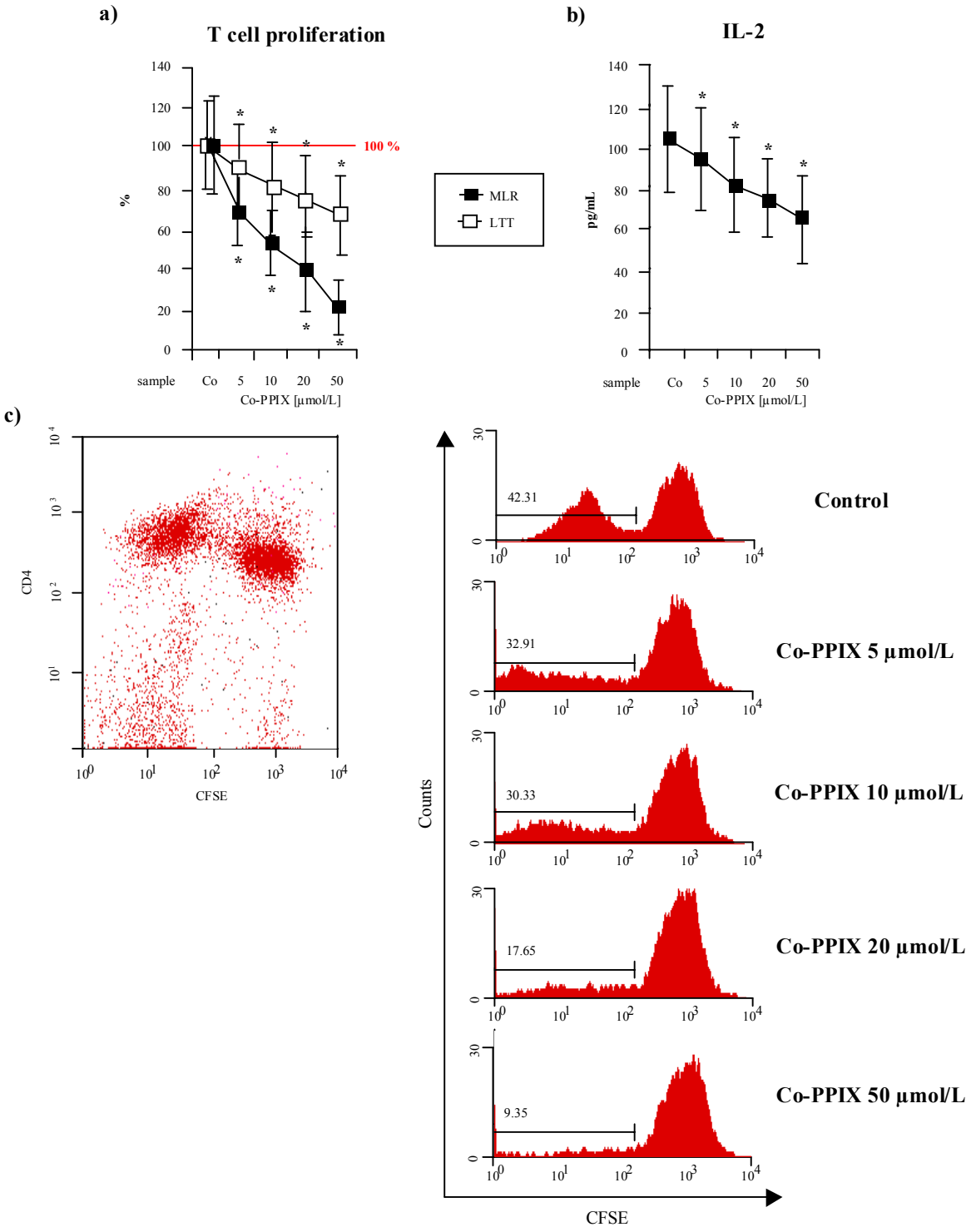


Figure 26. HO-1 induction by Co-PPIX inhibits the proliferation and IL-2 secretion of T-helper cells
a) Allogeneic human PBMC or PBMC and the recall antigen tetanus toxoid were cultured over 5 and 3 days in MLR and LTT, respectively, in the presence of vehicle (Co) or Co-PPIX. T cell proliferation from 9 independent experiments is given as mean ± SEM of the percentages of the values in control culture (MLR: 48,297±12,629 cpm; LTT: 46.391±1.787 cpm).
b) After MLR culture in the presence of vehicle (Co) or Co-PPIX, IL-2 concentrations were determined in supernatants. Data from 9 independent experiments are given as mean ± SEM.
c) Allogeneic responder PBMC were labeled with CFSE and cultured with allogeneic stimulator PBMC (mitomycin treated) over five days with vehicle (control) or different concentrations of Co-PPIX. Proportions of CD4+ T cells with diminished CFSE fluorescence were determined as measure for alloreactive T cell proliferation (right). Flowcytometric analysis of one representative out of 6 experiments is shown.
* $p<0.05$ vs. the respective control without Co-PPIX treatment, Wilcoxon test

3.2.2 Effects of HO-1 induction on human monocytes

3.2.2.1 Co-PPIX induces HO-1 mRNA expression in monocytes but not T cells

To clarify the mechanism of HO-1 induced immunosuppression, the induction of HO-1 in separated human immune cell populations was investigated. Based upon kinetic studies in PBMC (see 3.2.1.2), HO-1 mRNA expression in CD4 and CD8 positive T cells and in monocytes was determined at 6 hours after culture with Co-PPIX (Fig. 27a). HO-1 induction in T cells is not observed. In contrast, a dose-dependent increase in HO-1 mRNA expression is found in monocytes after Co-PPIX treatment. In accordance with these results, high HO-1 protein levels are exclusively detected in cell lysates from monocytes using HO-1-ELISA and Western-blot analyses (Fig. 27b).

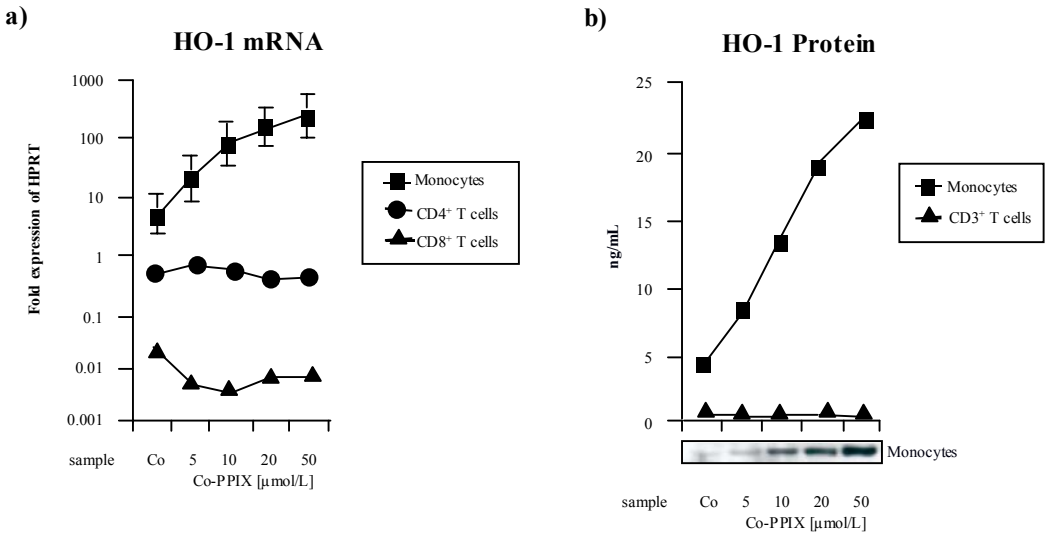


Figure 27. Co-PPIX induces HO-1 expression in monocytes and not in T cells
a) HO-1 mRNA expression in monocytes as well as in CD4+ and CD8+ T cells was determined after vehicle (Co) or Co-PPIX treatment for 6 hours. Data for monocytes are presented as mean ± SEM (logarithmic scale) of 4 independent experiments. For CD4+ and CD8+ T cells the results of one experiment are given.
b) After 6 hour treatment with vehicle (Co) or Co-PPIX, HO-1 protein expression was determined in cell lysates of monocytes and T cells with ELISA (top) and Western-blot (only monocytes are shown, bottom). Data from one experiment are given.

3.2.2.2 Co-PPIX induces HO-1 activity in monocytic cells

Bilirubin is released as an end-product of heme degradation by HO-1. It is reduced from biliverdin by biliverdin reductase. Bilirubin as indicator of enzymatic activity of HO-1 was determined in culture supernatants of Co-PPIX-treated cells. At 24 hours detectable levels of bilirubin are only found in supernatants of Co-PPIX-treated monocytes but not T cells (Fig.28a). In order to determine the kinetics of HO-1 enzymatic activity the monocytic THP-1 cell line was used. A dose-dependent increase of bilirubin levels is seen with a maximum at 30 hours (Fig. 28b).

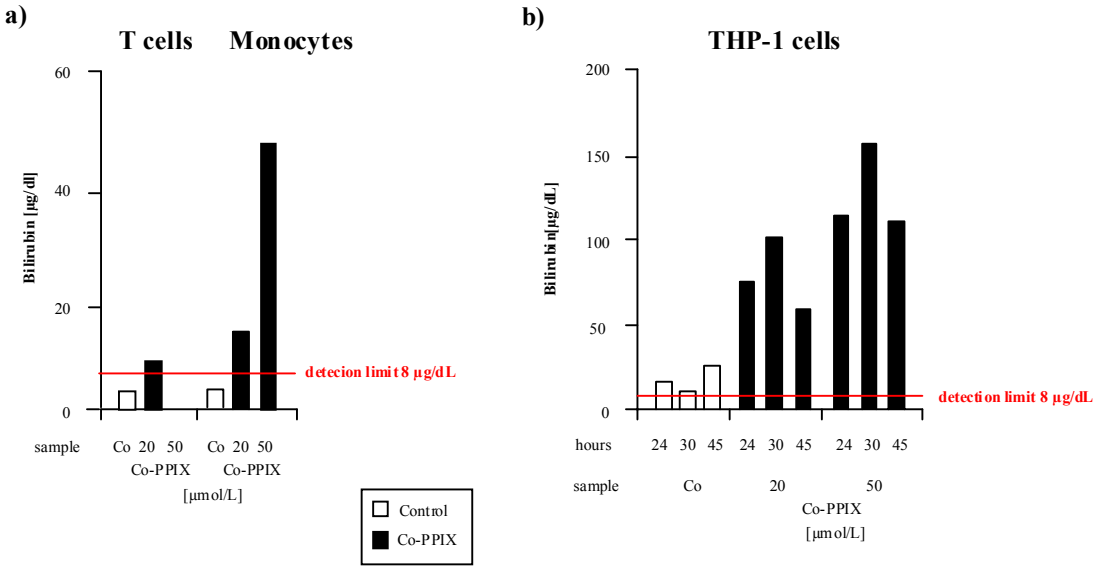


Figure 28. Co-PPIX induces bilirubin release in monocyte and THP-1 but not T cell cultures
a) Monocytes and T cells were treated with vehicle (white bars) or 20 and 50 µmol/L Co-PPIX (black bars) over 24 hours, and bilirubin concentrations in culture supernatants were determined. Data from one experiment are given.
b) THP-1 cells were cultured with vehicle (white bars) or 20 and 50 µmol/L Co-PPIX (black bars) over 24, 30 and 45 hours. Bilirubin concentrations in supernatants of one experiment are shown.

3.2.2.3 HO-1 induction inhibits accessory molecule expression on monocytes

The effects of HO-1 induction on immunophenotypic parameters of monocytes were flowcytometrically assessed after 24 hour Co-PPIX treatment. The monocytic expression of the MHC class II molecule, HLA-DR, and the costimulatory molecule, CD86, is significantly and dose-dependently inhibited by Co-PPIX treatment (Fig. 29a). Figure 29b shows the decrease of HLA-DR and CD86 expression on monocytes following Co-PPIX culture as a representative flow cytometric analysis in histograms.

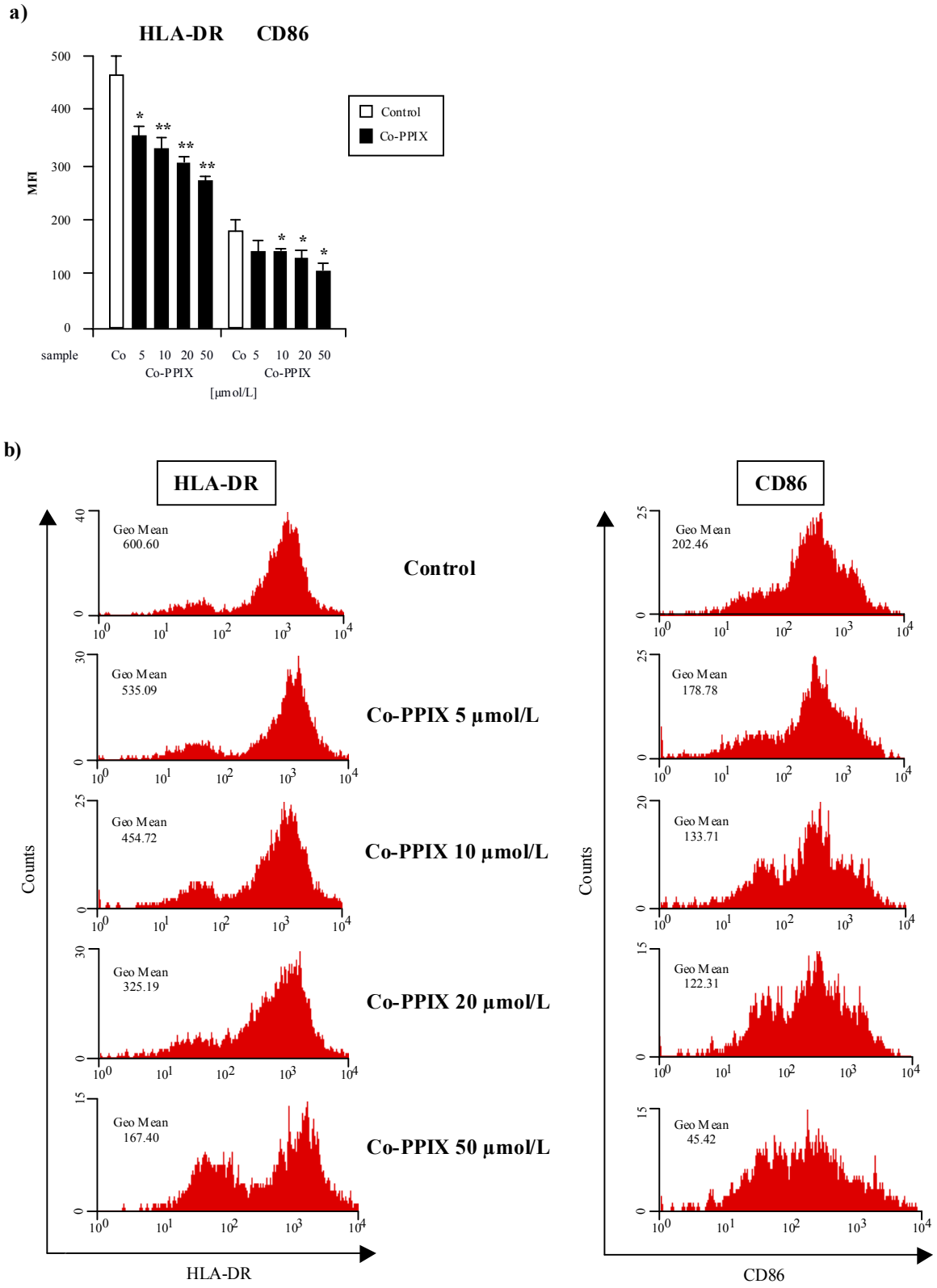


Figure 29. HO-1 induction by Co-PPIX inhibits accessory molecule expression on monocytes
a) The effect of HO-1 induction on monocytic HLA-DR and CD86 expression was determined by flowcytometry after 24 hour culture with vehicle (Co) or Co-PPIX. Mean fluorescence intensity (MFI) values are presented as mean ± SEM of 14 experiments.
*p<0.05, ** p<0.01 versus control culture without Co-PPIX, Wilcoxon test
b) Representative flowcytometric analysis of HLA-DR (left) and CD86 (right) inhibition by Co-PPIX.

3.2.2.4 HO-1 induction inhibits proinflammatory cytokine secretion and increases the secretion of IL-10

The effects of HO-1 induction on cytokine production were investigated at 24 hours after stimulation of monocytes with LPS (Fig. 30). The addition of Co-PPIX significantly and dose-dependently inhibited the secretion of the proinflammatory cytokines TNF-α, IL-12p70 and IL-18. In contrast, the secretion of the antiinflammatory cytokine IL-10 was dose-dependently increased. Thus, HO-1 induction by Co-PPIX shifts the cytokine profile of monocytes from a pro- to an antiinflammatory response.

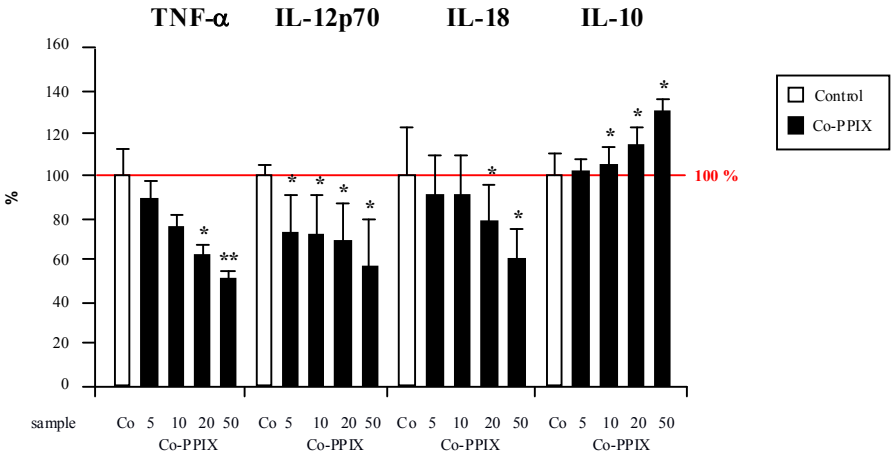


Figure 30. HO-1 induction by Co-PPIX inhibits proinflammatory cytokine secretion and increases the release of the antiinflammatory cytokine IL-10
Cytokine secretion of monocytes was determined after Co-PPIX treatment during 24 hour LPS (5 µg/mL) stimulation. Relative cytokine secretion in comparison to control culture (Co) is given as mean percentage ± SEM from 11 (TNF-α), 6 (IL-12p70), 7 (IL-18) and 8 (IL-10) independent experiments, respectively. Cytokine levels in control culture were: TNF-α: 4821±500 pg/mL; IL-12p70: 30±2 pg/mL; IL-18: 250±54 pg/ml; IL-10: 2999±26 pg/mL.
* $p<0.05$, ** $p<0.01$ versus control culture without Co-PPIX, Wilcoxon test

3.2.2.5 HO-1 induction increases the phagocytosis and the oxidative burst response of monocytes and granulocytes

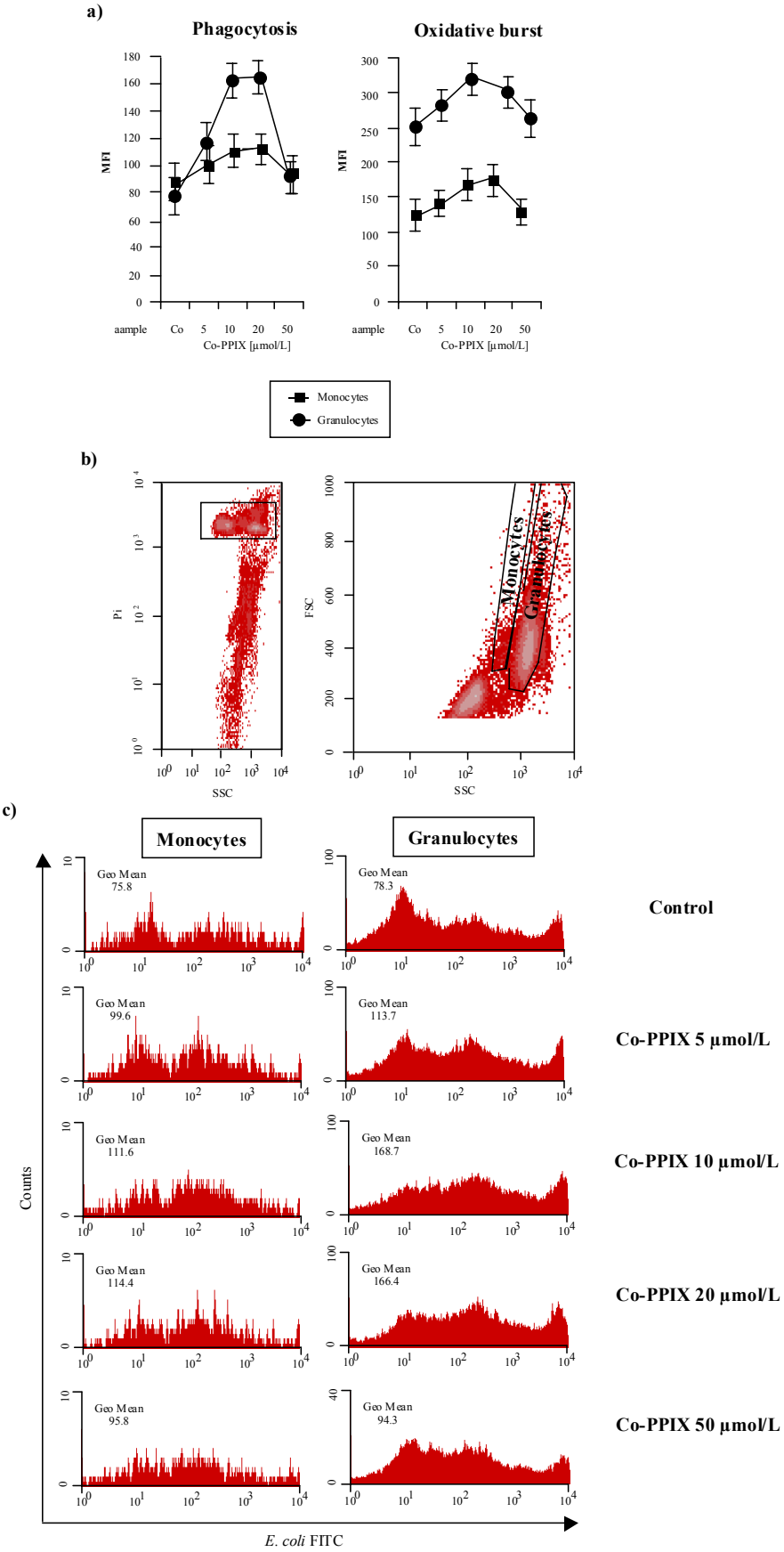
Finally the effect of Co-PPIX on the phagocytosis and oxidative burst activity of monocytes and granulocytes was investigated.

Phagocytosis is a process of active uptake of alive and dead materials by phagocytes (monocytes, macrophages and neutrophilic granulocytes) and plays an important role in the control of e.g. bacterial infection. Oxidative burst serves for the killing of uptaken materials by phagocytes by production of 0_2 radicals. Both processes remove the (bio)material from blood / tissue and usually abrogate the immune response.

The phagocytosis and the oxidative burst activity of blood phagocytes were flowcytometrically determined by exposure to *E. coli* bacteria in 18 hour whole blood culture. Phagocytosis by monocytes and granulocytes was dose-dependently increased at 5, 10 and 20 µmol/L Co-PPIX (Fig. 31a). Treatment with 50 µmol/L Co-PPIX decreases the phagocytosis to the values in control culture. Oxidative burst activity of monocytes and granulocytes exhibits a quite similar pattern.

Figure 31b shows an example of the monocyte and granulocyte gating for the phagocytosis and burst analysis. To exclude free bacteria, living cells were gated as strongly Pi-labeled cells (Fig. 31b, left). Cell size and granularity were used to gate monocytes and granulocytes (Fig. 31b, right).

The effect of Co-PPIX on phagocytosis, i.e. uptake of *E. coli*-FITC, is exemplarily shown in histograms for monocytes (Fig. 31c, left) and granulocytes (Fig. 31c, right). Figure 31d demonstrates the influence of Co-PPIX on *E. coli*-triggered oxidative burst activity in monocytes (left panel) and granulocytes (right panel). The induction of oxidative burst leads to the intracellular generation of oxidized fluorogenic rhodamine.


a)
Phagocytosis
Oxidative burst
MFI
aample
Co
5
10
20
50
Co-PPIX [µmol/L]
Monocytes
Granulocytes
b)
Pi
SSC
FSC
Monocytes
Granulocytes
c)
Monocytes
Granulocytes
Geo Mean
75.8
78.3
Control
99.6
113.7
Co-PPIX 5 µmol/L
111.6
168.7
Co-PPIX 10 µmol/L
114.4
166.4
Co-PPIX 20 µmol/L
95.8
94.3
Co-PPIX 50 µmol/L
Counts
E. coli FITC

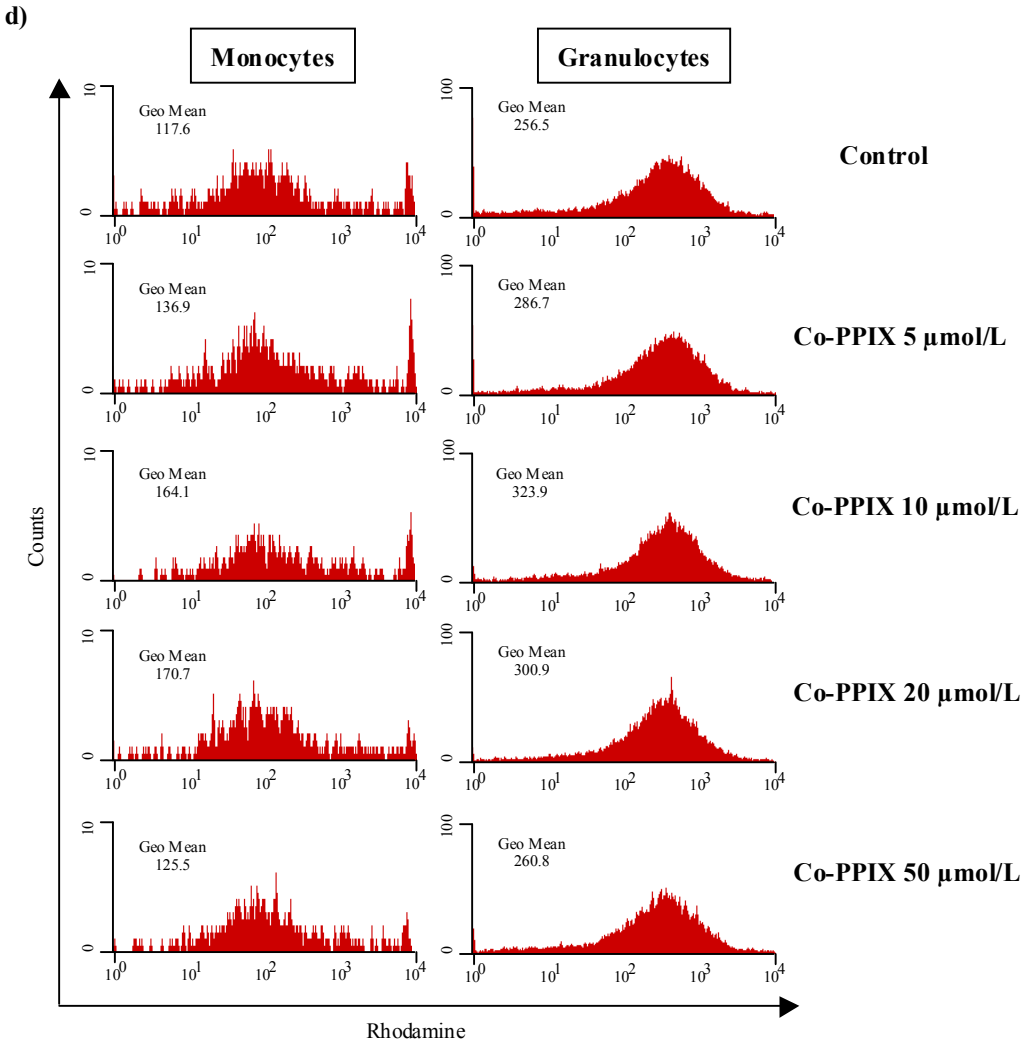


Figure 31 HO-1 induction by Co-PPIX increases phagocytosis and oxidative burst activity of monocytes and granulocytes
Whole blood was cultured over 18 hours with different concentrations of Co-PPIX or vehicle (Co) and analyzed by flow cytometry.
a) Phagocytosis (left) and oxidative burst activity (right) in monocytes and granulocytes from three healthy volunteers are shown as mean ± SEM of mean fluorescence intensity (MFI).
b) An exemplary flowcytometric analysis showing the gating of phagocytes in whole blood. Addition of propidium iodide (Pi) leads to discrimination of bacteria and gated Pi strong-positive nucleated human cells (left). Monocytes and granulocytes are distinguished by size (FSH) and granularity (SSC) (right dot plot).
c) Fluorescence histograms exemplifying the effect of Co-PPIX on E. coli-FITC uptake by monocytes and granulocytes.
d) Fluorescence histograms demonstrating the effect of Co-PPIX on E. coli-triggered rhodamine generation in monocytes and granulocytes.

In summary, HO-1 induction by Co-PPIX leads to the suppression of MHC II (HLA-DR) and costimulatory (CD86) molecule expression on monocytes which is necessary for an adequate antigen-presenting activity. The secretion of proinflammatory and immunostimulatory monokines is inhibited whereas the secretion of the antiinflammatory cytokine IL-10 is increased. In contrast, phagocytosis and oxidative burst activity, which are important for scavenging and killing of biomaterial and for the abrogation of an immune reaction, were strengthened by HO-1 induction.

3.2.3 Effect of HO-1 induction on monocyte-derived dendritic cells (MDDC)

3.2.3.1 Generation of MDDC from human blood monocytes

Dendritic cells can be differentiated from blood monocytes by 7 day culture with GM-CSF and IL-4. The evolving immature monocyte-derived dendritic cells (iMDDC) show a downregulation of CD14 expression, a minimal expression of CD83 and a high expression of CD1a. The expression of HLA-DR and CD86 is increased. A subsequent stimulation with LPS for three days leads to the maturation of iMDDC to mature monocyte-derived dendritic cells (mMDDC) with a strong antigen-presenting capacity. These potent immunostimulatory cells are characterized by high expression of CD83 (typical marker of DC maturation) as well as HLA-DR and CD86. The expression levels of CD14 (marker for monocytes/macrophages) and CD1a (iMDDC marker) are strongly reduced. Figure 32 demonstrates the generation of iMDDC and mMDDC from monocytes on the basis of alterations of these immunophenotypic parameters.

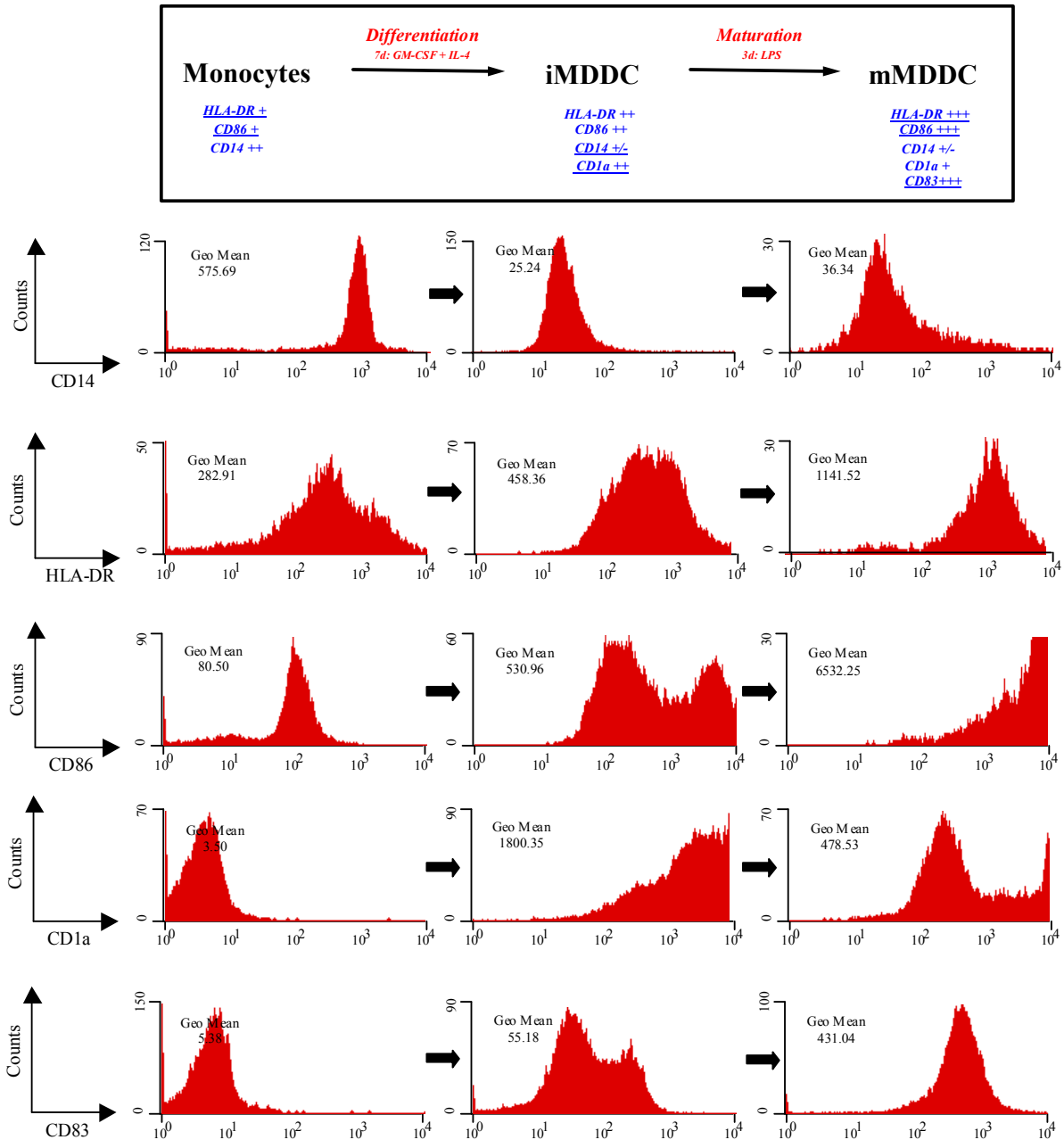


Figure 32. Immunophenotypic changes during the generation of iMDDC and mMDDC from blood monocytes
Monocytes, iMDDC (generated by 7 day incubation with GM-CSF and IL4) and mMDDC (yielded by subsequent LPS stimulation for 3 days) were flowcytometrically characterized regarding the expression of CD14, HLA-DR, CD86, CD1a and CD83. One representative analysis is given as histograms and mean fluorescence intensity values (GeoMean).

3.2.3.2 Co-PPIX induces HO-1 expression in immature and mature MDDC

To provide the mechanistic base for further investigations, HO-1 mRNA and protein induction by Co-PPIX treatment were investigated in monocytes and MDDC. Figure 33a shows the dose-dependent HO-1 mRNA induction in monocytes, iMDDC and mMDDC after 6 hour Co-PPIX treatment. Interestingly, pronounced HO-1 protein induction was detected only in cell lysates of monocytes and iMDDC but not mMDDC (Fig. 33b).

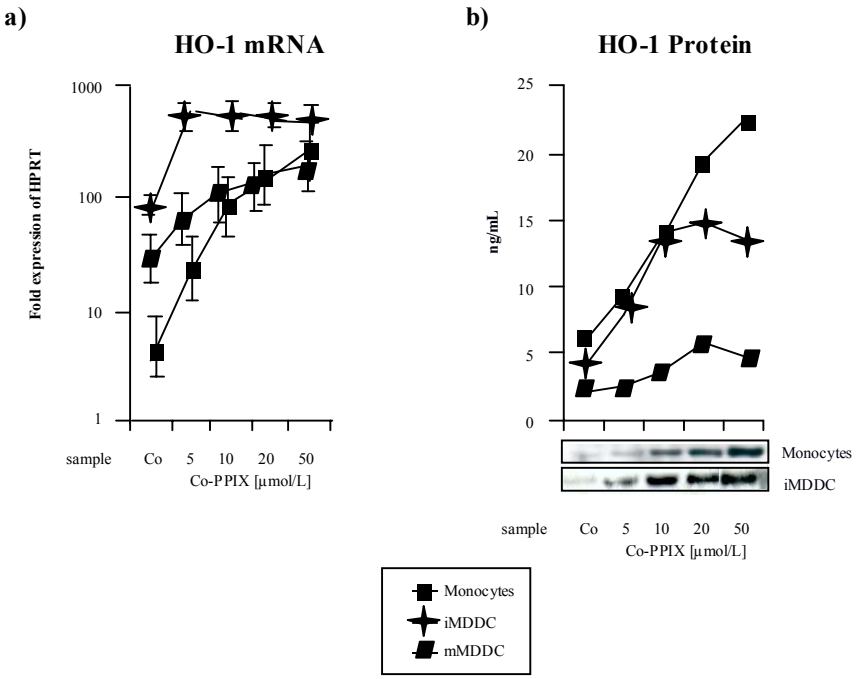
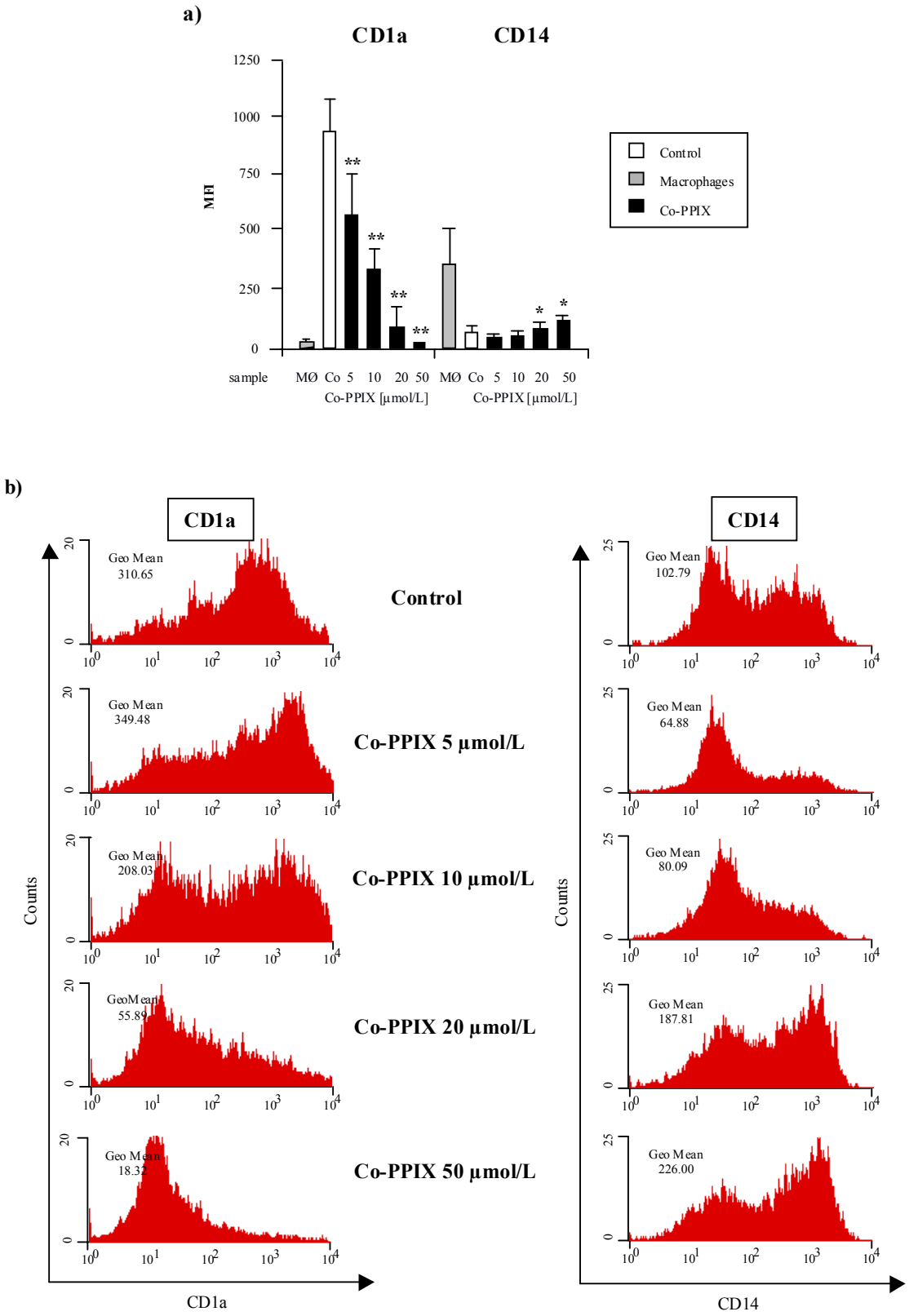


Figure 33. HO-1 mRNA induction in antigen presenting cells
a) HO-1 mRNA expression in monocytes, immature MDDC (iMDDC) and mature MDDC (mMDDC) was determined after vehicle (Co) or Co-PPIX treatment for 6 hours. Data for monocytes and MDDCs are presented as mean ± SEM (logarithmic scale) of 4 independent experiments.
b) After 6 hour treatment with vehicle (Co) or Co-PPIX, HO-1 protein expression was determined in cell lysates of monocytes, immature and mature MDDC by ELISA (top) and Western-blot analyses (only monocytes and iMDDC are shown, bottom), respectively. Data from one experiment are given.

3.2.3.3 HO-1 induction inhibits the differentiation of immature MDDC from monocytes

Co-PPIX treatment during the DC differentiation culture of monocytes (7 days with GM-CSF and IL-4) prevented the increase of CD1a expression in a dose-dependent manner, whereas the expression of CD14 was increased (Fig. 34a). These data suggest that HO-1 induction inhibits DC differentiation and instead promotes the transition into a more "macrophage-like" status. These data correlate with the Co-PPIX-triggered promotion of phagocytosis and oxidative burst of phagocytes, which is dependent on high expression of CD14.

Figure 34b demonstrates the inhibition of CD1a and the induction of CD14 expression in an exemplary flowcytometric analysis. The induced expression of the accessory molecules HLA-DR and CD86 is also inhibited by Co-PPIX (Fig. 34c).


a)
CD1a
CD14
MFI
1250
1000
750
500
250
0
Control
Macrophages
Co-PPIX
sample
MØ Co 5 10 20 50 MØ Co 5 10 20 50
Co-PPIX [µmol/L]
Co-PPIX [µmol/L]
b)
CD1a
CD14
Control
Co-PPIX 5 µmol/L
Co-PPIX 10 µmol/L
Co-PPIX 20 µmol/L
Co-PPIX 50 µmol/L
Geo Mean 310.65
Geo Mean 102.79
Geo Mean 349.48
Geo Mean 64.88
Geo Mean 208.03
Geo Mean 80.09
GeoMean 55.89
Geo Mean 187.81
Geo Mean 18.32
Geo Mean 226.00
Counts
CD1a
CD14

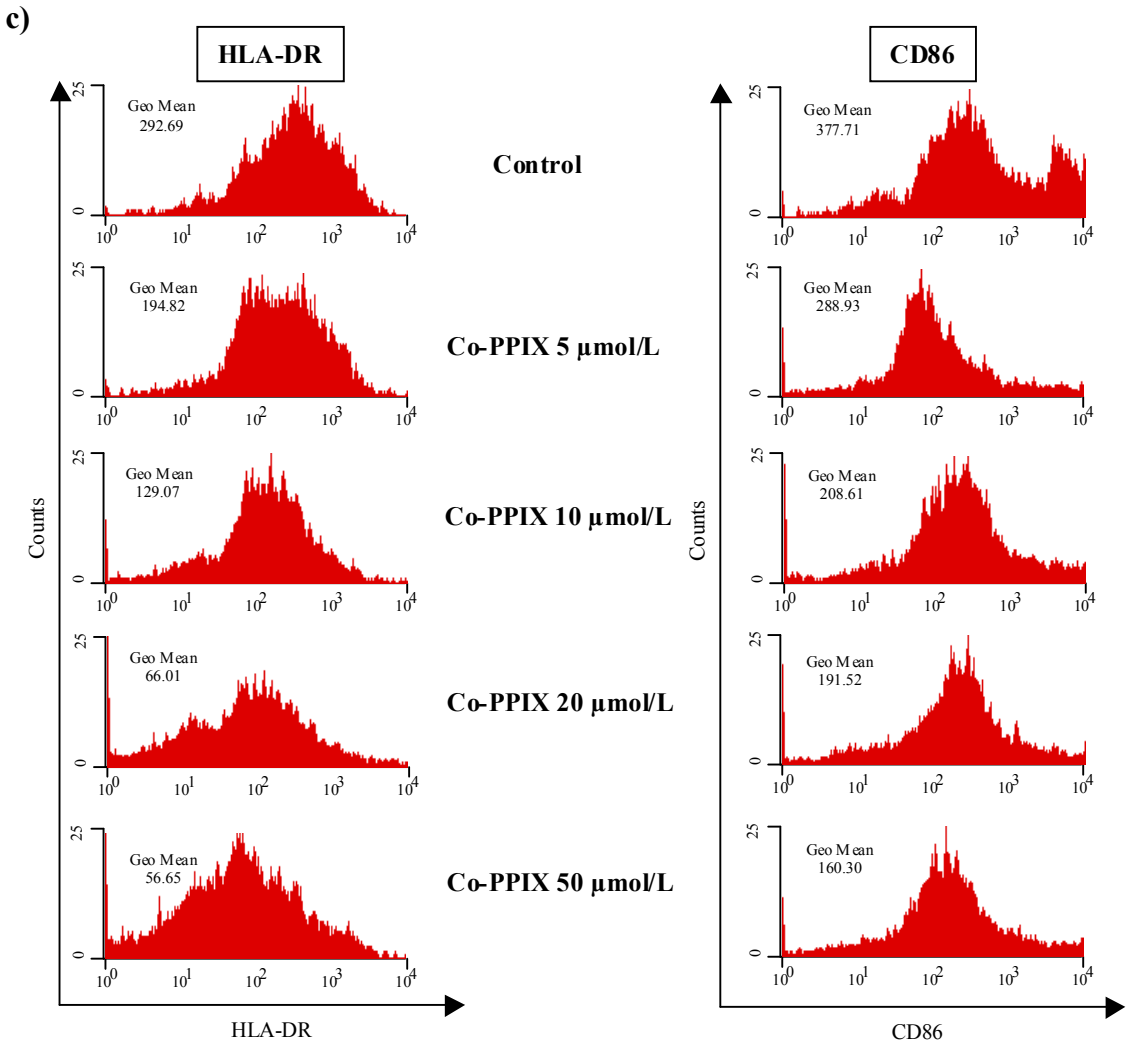
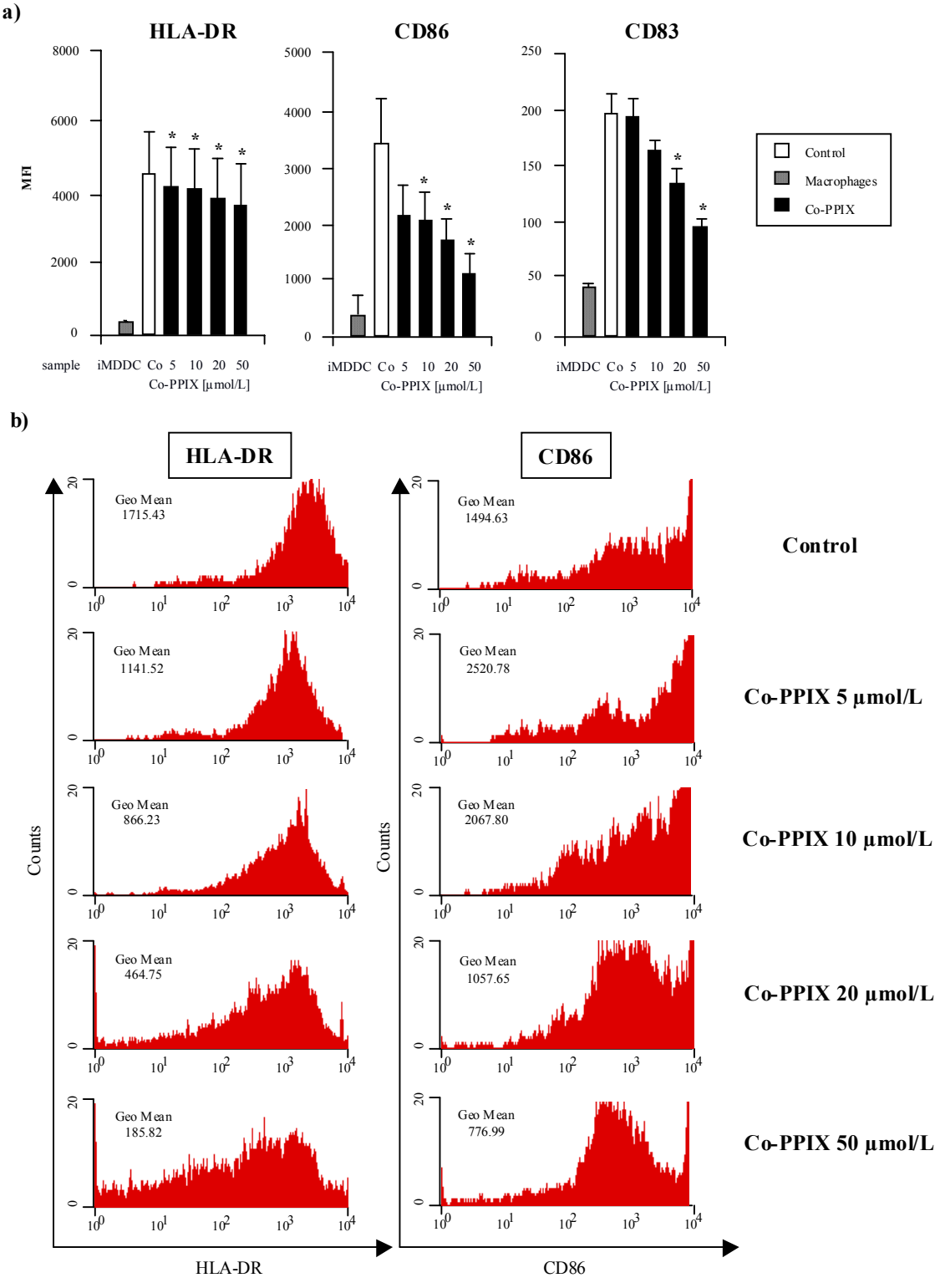


Figure 34. HO-1 induction by Co-PPIX inhibits the differentiation of MDDC
a) The effect of HO-1 induction on MDDC differentiation was flowcytometrically determined by analysis of CD1a and CD14 expression after addition of Co-PPIX to the 7 day differentiation culture with GM-CSF and IL-4. For comparison the expression values of monocyte-derived macrophages after 7 day culture with M-CSF are shown (M∅). Mean fluorescence intensities are given as mean ± SEM from 6 independent experiments.
* $p<0.05$, ** $p<0.01$ versus the control culture without Co-PPIX, Wilcoxon test
b) and c) Flowcytometric analyses of CD14, CD1a (b), HLA-DR and CD86 expression (c) after Co-PPIX treatment during the MDDC differentiation culture from one representative experiment are shown.

3.2.3.4 HO-1 induction suppresses the maturation of MDDC

Our next intention was to study the effect of Co-PPIX on the phenotypic maturation and function of MDDC. Because of the crucial role of mature DCs for T cell immunity, the interference with DC maturation might represent the most relevant mechanism for the *in vivo* inhibition of T cell immunity by an enhanced HO-1 activity.

The effect of HO-1 induction on MDCC maturation was determined by addition of Co-PPIX to the maturation culture (LPS stimulation of iMDDC for 3 days). Co-PPIX dose-dependently inhibited the increases in the expression of the accessory molecules, HLA-DR and CD86, as well as of the DC-maturation marker CD83 (Fig. 35a). These data indicate that an enhanced HO-1 activity counteracts the phenotypic maturation of dendritic cells. Additionally, CD1a expression is also inhibited and the expression of CD14 is strongly induced. Exemplary fluorescence histograms for the dose-dependent effects of Co-PPIX on HLA-DR, CD86 and CD83 as well as on CD1a and CD14 expression are shown in Figure 35 b-d.


a)
HLA-DR
CD86
CD83
MFI
8000
6000
4000
2000
0
5000
4000
3000
2000
1000
250
200
150
100
50
sample iMDDC Co 5 10 20 50
Co-PPIX [µmol/L]
Control
Macrophages
Co-PPIX
b)
HLA-DR
CD86
Geo Mean 1715.43
Geo Mean 1494.63
Geo Mean 1141.52
Geo Mean 2520.78
Geo Mean 866.23
Geo Mean 2067.80
Geo Mean 464.75
Geo Mean 1057.65
Geo Mean 185.82
Geo Mean 776.99
Counts
Control
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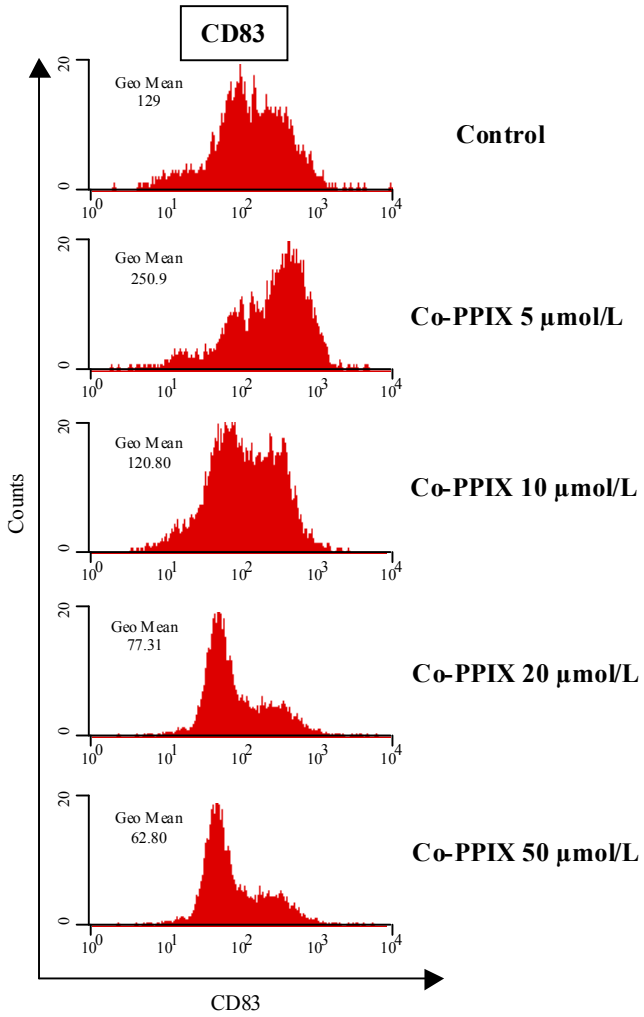
Co-PPIX 20 µmol/L
Co-PPIX 50 µmol/L
HLA-DR
CD86

c)


CD83
Geo Mean
129
Control
Geo Mean
250.9
Co-PPIX 5 µmol/L
Geo Mean
120.80
Co-PPIX 10 µmol/L
Geo Mean
77.31
Co-PPIX 20 µmol/L
Geo Mean
62.80
Co-PPIX 50 µmol/L
Counts
CD83

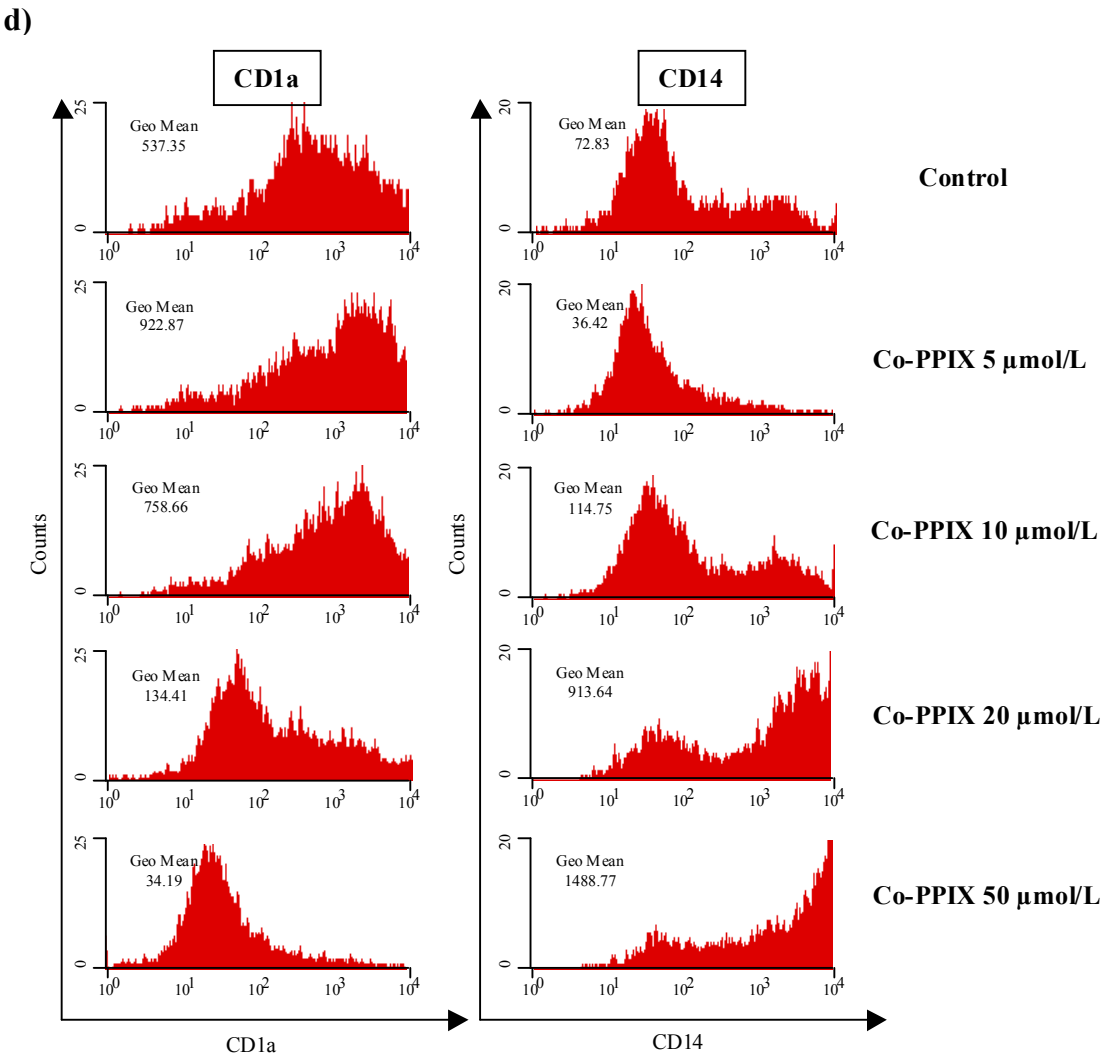


Figure 35. HO-1 induction by Co-PPIX suppresses the maturation of MDDC
a) Effect of HO-1 induction on MDDC maturation was assessed by determination of HLA-DR, CD86 and CD83 expression after addition of Co-PPIX to the 3 day maturation culture with LPS. For comparison the expression pattern of the immature MDDCs (iMDDC) is given. Mean fluorescence intensity (MFI) values are presented as mean ± SEM from 7 independent experiments.
* $p<0.05$ versus control culture without Co-PPIX, Wilcoxon test
b)-d) One representative flowcytometric analysis of HLA-DR, CD86 (b), CD83 (c) as well as CD1a and CD14 (d) expression after Co-PPIX treatment during the MDDC maturation culture is shown.

3.2.3.5 HO-1 induction shifts the cytokine profile of MDDC to an antiinflammatory profile

The effect of HO-1 induction on cytokine production of MDDC was determined by Co-PPIX treatment during a 24 hour stimulation culture of iMDDC with LPS (Fig. 36a). Co-PPIX treatment almost completely inhibited the secretion of IL-12p40 and IL-12p70 and, to a lesser degree, TNF-α. In contrast, the secretion of the antiinflammatory cytokine IL-10 was dose-dependently increased. Interestingly, determination of cytokine mRNA expression revealed an inhibition for all cytokines indicating that the increase of IL-10 production is regulated on post-transcriptional level (Fig. 36b).

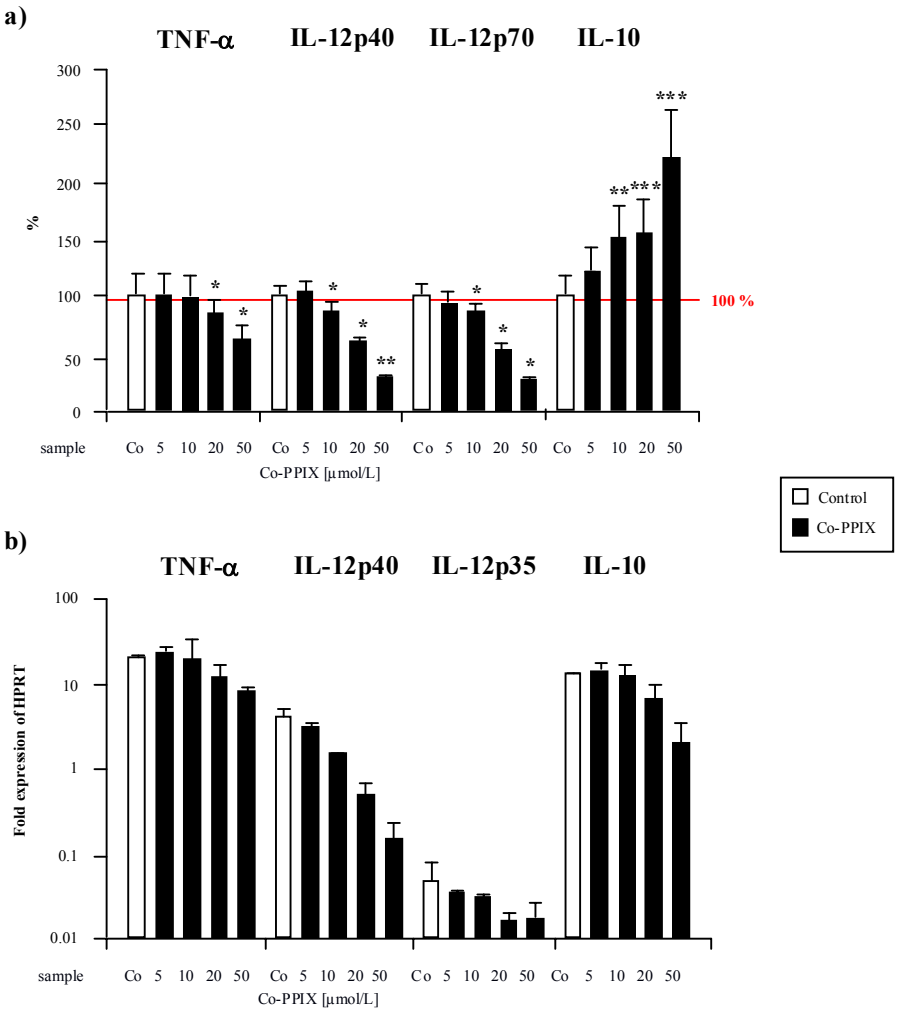


Figure 36. HO-1 induction by Co-PPIX decreases proinflammatory cytokine production of MDDC and increases IL-10 protein
a) Cytokine secretion of iMDDC was determined after Co-PPIX treatment during 24 hour LPS stimulation. Relative cytokine secretion in comparison to control culture is given as mean percentage values ± SEM from 10 (IL-12p40, IL-12p70) and 6 independent experiments (TNF-α, IL-10), respectively. Cytokine levels in control culture were: TNF-α: 8804±1658 pg/mL; IL-12p40: 1299±110 pg/mL; IL-12p70: 37±4 pg/mL; IL-10: 2140±342 pg/mL.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus control culture, Wilcoxon test
b) Effect of Co-PPIX treatment on cytokine mRNA expression was determined by real-time RT-PCR after 6 hours LPS stimulation. Relative expression of TNF-α, IL-12p40, IL-12p35, and IL-10 in comparison to the house-keeping gene HPRT is shown in logarithmic scale as mean ± SEM of two independent assays.

In summary, HO-1 induction by Co-PPIX shifts the cytokine profile of MDDC from a proinflammatory and immunostimulatory to an antiinflammatory and immunosuppressive response.

3.2.3.6 HO-1 induction inhibits the antigen-presenting capacity of MDDC

The effect of HO-1 induction on antigen-presenting capacity of mature MDDC was investigated in MLR with allogeneic T-helper (Th) cells and in LTT with autologous Th cells and the recall antigen tetanus toxoid. Co-PPIX treatment provoked a dose-dependent inhibition of Th cell proliferation and IL-2 secretion (Fig. 37a-b).

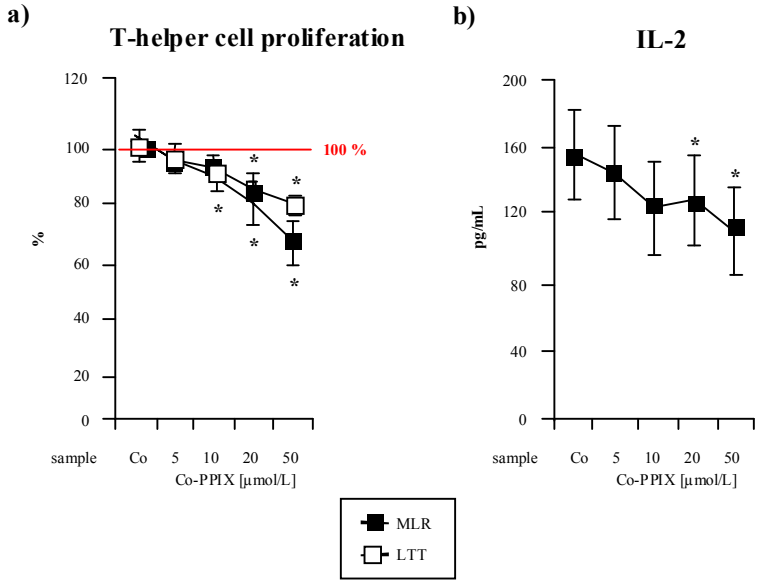


Figure 37. HO-1 induction by Co-PPIX inhibits antigen presenting capacity of MDDC
Mature MDDC were cultured either with allogeneic Th cells in MLR for 5 days or with autologous Th cells and tetanus toxoid in LTT for 3 days in the presence of Co-PPIX.
a)Relative Th cell proliferation in comparison to control cultures (MLR: 116,977±5,600 cpm; LTT: 103,676±1000 cpm) are given as mean percentage values ± SEM of 9 (MLR) and 7 (LTT) independent experiments, respectively.
b)IL-2 concentrations in culture supernatants of MLR are shown as mean ± SEM of 9 independent experiments.
* $p<0.05$ versus control culture, Wilcoxon test

3.2.4 Effect of adenoviral HO-1 transduction on monocyte-derived dendritic cells

3.2.4.1 Transduction efficiency

In order to determine whether enhanced HO-1 activity is indeed responsible for the observed effects of Co-PPIX treatment, adenoviral transductions of immature MDDC either with HO-1 (Ad-vector-HO-1) or a control vector (Ad-vector-zero) were performed. Controls with AdGFP indicated a transduction efficiency of about 90% at 24 hours in iMDDC (Fig. 38a). In order to induce DC maturation, the transduced iMDDC were stimulated with LPS for 3 days. Thereafter, the AdGFP detection in mMDDC was about 80 % (Fig. 38b).

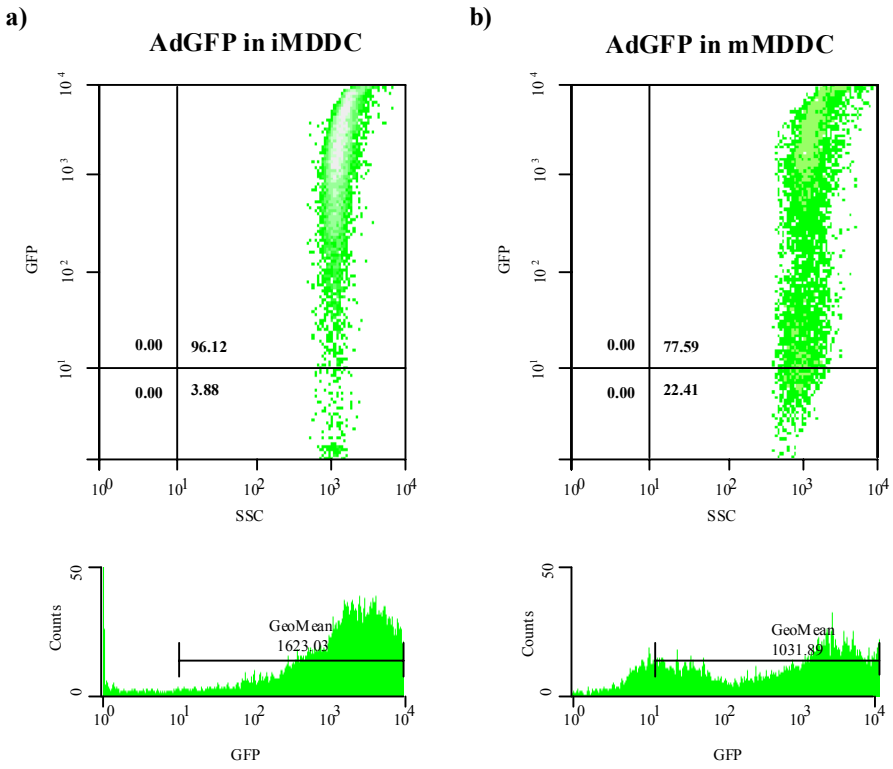


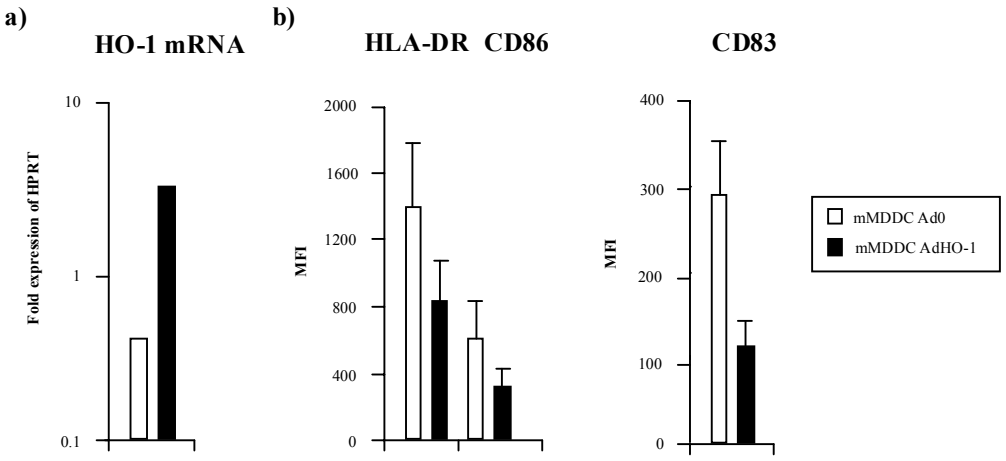
Figure 38. Efficacy of adenoviral GFP transduction in iMDDC
a) iMDDC were transduced with AdGFP over 3 hours. After 24 hours the GFP expression was determined. Flowcytometric analysis from one representative experiment is shown.
b) For maturation transduced iMDDC were activated with LPS for 3 days. Thereafter the GFP fluorescence was determined. One representative experiment is shown.

3.2.4.2 HO-1 transduction inhibits MDDC maturation and function

In HO-1 transduced MDDC an increased HO-1 expression was observed at the end of the maturation culture (Fig. 39a).

Similar to the results obtained with Co-PPIX treatment during MDDC maturation, the expression of HLA-DR, CD86 and CD83 is inhibited in HO-1-transduced cells (Fig 39b). Figure 39c shows an exemplary flowcytometric analysis of HLA-DR, CD86 and CD83 expression as histograms.

Moreover, functional relevance of these alterations is demonstrated by the diminished capacity of HO-1-transduced MDDC to stimulate the proliferation of allogeneic Th cells in mixed leukocyte reaction (Fig. 39d).



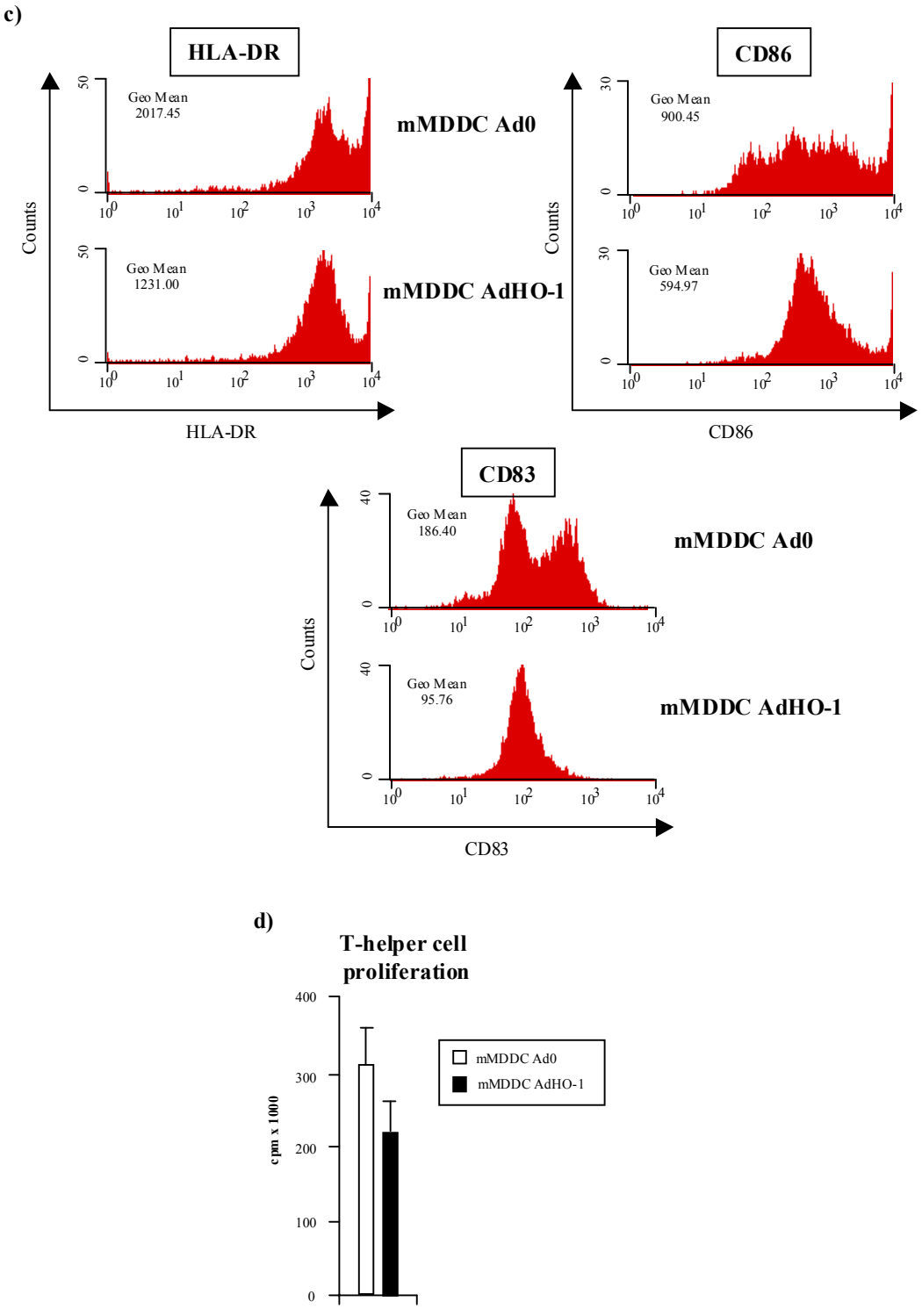


Figure 39. Adenoviral HO-1 transduction impairs the maturation and function of MDDC
Immature MDDC were adenovirally transduced with either AdHO-1- or Ad0-vector and thereafter stimulated for 3 days with LPS for induction of maturation.
a) HO-1 mRNA expression was assessed after the maturation culture of MDDC. Results of one experiment are given.
b) Expression of HLA-DR, CD86 and CD83 was determined by flowcytometry after maturation culture of transduced iMDDC. Mean fluorescence intensities (MFI) are shown as mean ± SEM from 4 independent experiments.
c) Flowcytometric analysis of HLA-DR, CD86 and CD83 expression is given for one representative experiment of Ad0 and AdHO-1 transduced MDDC.
d) Antigen-presenting capacity of adenovirally transduced MDDC was assessed by MLR with allogeneic Th cells. Th cell proliferation is given as mean ± SEM from 4 independent experiments.

Overall, these data strongly support the conclusions that the effects observed with the tool compound Co-PPIX are mediated by induction of HO-1 and that an enhanced HO-1 activity impairs T cell immunity via the interference with the maturation and function of dendritic cells.

4 Discussion

Heme oxygenase 1 is an antiinflammatory enzyme, which is induced by several cellular stress situations and by various drugs. An induction by cellular stress leads primarily to an inflammation, which is characterized by secretion of proinflammatory cytokines, and the inflammation by itself induces secondarily the HO-1. Carbon monoxide (CO) and biliverdin / bilirubin, the end-products of heme degradation by heme oxygenases, lead then to inhibition of inflammation. This inhibition is often to low and to late, therefore the tissue injury is limited but not resolved.

The HO-1 knockout mice and transfection experiments confirmed the high relevance of HO-1 for the regulation of the immune system. Additionally, various synthetic and natural molecules have been described, which induce HO-1 primarily without concomitant cell injury and inflammation. Co-PPIX is a strong inducer of HO-1, whose antiinflammatory and cytoprotective effects have been proven in different *in vivo* and *in vitro* assays (Amersi et al, 1999; Clark et al, 2000b; DeBruyne et al, 2000; Liu et al, 2001; Shan et al, 2000; Woo et al, 1998; Woo et al, 2000).

Protective effects of HO-1 induction in models for inflammatory tissue injury are manifold published, however, the underlying mechanisms are poorly understood. We demonstrated for the first time the physiological role of HO-1 for the limitation of T cell-dependent skin inflammation and the antiinflammatory and immunosuppressive effects of HO-1 induction on cutaneous inflammation. Moreover, we got strong evidence that the immunosuppressive *in vivo* and *in vitro* effects of enhanced HO-1 activity are related to its effects in antigen-presenting cells. So, HO-1 induction or transduction potently inhibits the maturation and function of dendritic cells.

In detail the following results were yielded in this study:

1) HO-1 expression is increased in chronic skin inflammation in psoriasis and is rapidly induced in an acute T cell-dependent skin inflammation model in mice. In the latter HO-1 expression is mainly observed in infiltrating immune cells.
2) An inhibitor of HO-1 activity, Sn-PPIX, augments experimental skin inflammation demonstrating the physiological role of HO-1 for the limitation of cutaneous inflammation.
3) Co-PPIX strongly induced HO-1 mRNA expression and activity in mice *in vivo* and inhibits murine contact hypersensitivity response both when applied before antigen challenge or around sensitization.
4) Co-PPIX treatment suppresses T cell immunity in an *ex vivo* assay (MLR) after treatment of mice and *in vitro* in assays with human immune cells (MLR and LTT).

5) Co-PPIX triggers HO-1 expression and activity preferentially in antigen-presenting cells but not T cells.
6) HO-1 induction by Co-PPIX shifts the proinflammatory and immunostimulatory cytokine profile of monocytes and dendritic cells to an antiinflammatory profile while augmenting the phagocytosis and respiratory burst response of monocytes.
7) HO-1 induction by Co-PPIX and adenoviral HO-1 transduction inhibit the maturation of monocyte-derived dendritic cells (MDDC) and decrease their antigen-presenting capacity.

In conclusion, antigen-presenting cells, especially DC, are a major target for HO-1 leading to inhibition of the adaptive immune response. The pharmacological enhancement of HO-1 activity may therefore represent a potent approach for the immunotherapy of T cell-dependent inflammatory diseases including cutaneous inflammation (e.g. psoriasis).

4.1 HO-1 is increased in cutaneous inflammation and limits the inflammatory response

4.1.1 HO-1 expression in T cell-dependent skin inflammation

HO-1 mRNA is physiologically enhanced in lesional skin of psoriasis patients. These results confirm data from Hanselmann et al. who described increased HO-1 mRNA and protein expression in psoriatic skin and in injured skin of mice. They postulated a relevant role of HO-1 in wound healing (Hanselmann et al, 2001). Similarly, an increased HO-1 expression in normal and diabetes-impaired wound repair in mice was published by Kämpfer and co-workers (Kampfer et al, 2001).
In contrast to the increased HO-1 expression in lesional skin no alterations were observed in peripheral blood (whole blood and separated T cells and monocytes) of psoriasis patients. One reason for this might be that activated immune cells with higher HO-1 expression are sequestrated to the lesional skin. Similar expression differences between inflamed tissue and blood immune cells have also been described for IL-18 expression in rheumatoid arthritis (Moller et al, 2002).

Investigation of HO-1 expression in human allergic contact dermatitis (nickel reaction) did not show any HO-1 regulation in the skin. This might be due to the rather late skin sampling (24 hours) after acute challenge and the investigation of only HO-1 mRNA, not protein expression. So, in acute DNFB-induced contact hypersensitivity in mice a significant 5-fold increase of HO-1 expression at 3 hours after DNFB challenge was demonstrated. In skin sections high expression of HO-1, I-A/I-E and neutrophil protein was contemporary observed at 24 hours after DNFB challenge. The absence of significant T cell detection indicates that HO-1 is rather expressed in infiltrating non-T cells.

Several reports indicate an increase of HO-1 expression especially in monocytes and macrophages. So,

HO-1 induction has been reported in alveolar macrophages of mice in allergic airway inflammation (Kitada et al, 2001), in macrophages and circulating monocytes from patients after coronary artery bypass graft surgery (Philippidis et al, 2004), and in murine macrophages after IL-10 treatment (Lee et al, 2002). Kampfer and colleagues showed HO-1 in infiltrating monocytic cells in injured skin of mice (Kampfer et al, 2001). These data indicate that antigen-presenting cells / macrophages represent a main source of physiologically enhanced HO-1 expression in *in vivo* inflammation.

4.1.2 HO-1 inhibition augments T cell-dependent skin inflammation

The physiologic role of enhanced HO-1 expression in T cell-dependent skin inflammation was characterized by treatment with the HO-1 inhibitor Sn-PPIX (Blumenthal et al, 2005) in acute DNFB-induced contact hypersensitivity model. The dose-dependent increase of ear inflammation demonstrates that the enhanced endogenous HO-1 expression limits the cutaneous inflammatory response.
An increase in inflammation by HO-1 inhibition is published in different mouse models and emphasized by data from HO-1 knockout mice and a HO-1 deficient patient (Koyamada et al, 1998; Poss et al, 1997a and 1997b; Sato et al, 2001; Soares et al, 1998; Soares et al, 2001; Wiesel et al, 2000).

4.2 Pharmacological HO-1 induction inhibits T cell-dependent skin inflammation and enhances systemic levels of bilirubin

4.2.1 Co-PPIX suppresses T cell-dependent skin inflammation

For assessing pharmacological HO-1 superinduction as antiinflammatory approach for T cell-dependent skin inflammation, we used the well known strong HO-1 inducer Co-PPIX in the DNFB- and TMA-induced contact hypersensitivity models, which are characterized by a dominant type 1 and type 2 cytokine pattern, respectively.

Co-PPIX treatment potently inhibited T cell-dependent skin inflammation both when applied around sensitization or before hapten challenge. Moreover, concurrent treatment with the HO-1 activity inhibitor Sn-PPIX, used to ensure the HO-1 specificity of effects, prevented the antiinflammatory action of Co-PPIX in DNFB model.

This first demonstration of antiinflammatory effects of HO-1 induction in T cell-dependent skin inflammation is in accordance with literature data demonstrating HO-1-mediated suppression of T cell immunity in models for allergic encephalitis, inflammatory bowel diseases, rheumatoid arthritis, asthma, and allotransplantation (Guo et al, 2001; Hildebrandt et al, 2003; Kitada et al, 2001; Maines, 2002; Woo et al, 1998).

4.2.2 Systemic effects of Co-PPIX treatment

Co-PPIX treatment of mice leads to a strong HO-1 induction in skin, liver, lung, kidney and lymph nodes that was associated with an increase of systemic bilirubin levels, as indicator of HO-1 enzyme activity. Similar findings were reported by Woo (Woo et al, 1998). It is likely, therefore, that such a systemic increase of products of the HO-1 enzymatic pathway has paracrine and endocrine effects in addition to direct cellular effects in the cells in which HO-1 is induced. Thus, treatment with carbon monoxide or biliverdin, the natural products of heme catabolism, were found to suppress the proliferation and IL-2 secretion of T cells similar to HO-1-transfection and to prolong allograft survival (Akamatsu et al, 2004; Pae et al, 2003 and 2004; Song et al, 2004; Yamashita et al, 2004). Moreover, carbon monoxide-treated macrophages showed a decreased LPS-triggered production of TNF-α and IL-1β whereas the secretion of IL-10 was increased (Otterbein et al, 2000).

4.3 HO-1 induction inhibits T cell immunity in ex vivo assay in mice and in vitro assays in human immune cells

4.3.1 Ex vivo MLR with mice splenocytes

Concluding from our *in vivo* data we investigated the effect of Co-PPIX on standard assays for T cell immunity. The alloreactive proliferation of spleen cells from Co-PPIX treated C57Bl/6 and Balb/c mice was significantly inhibited in *ex vivo* MLR. Interestingly, stronger effects were observed by Co-PPIX treatment of donor mice for the stimulator cells, which are responsible for antigen-presentation, than of the responder splenocyte donors. This indicates that *in vivo* HO-1 induction rather affects antigen-presenting cells than T cells.

4.3.2 In vitro MLR and LTT with human PBMC

When looking for the effect of Co-PPIX on human *in vitro* assays for T cell immunity we demonstrated an inhibition of lymphocyte proliferation in MLR with allogeneic PBMC and in LTT in response to the recall-antigen tetanus toxoid. Additionally the IL-2 secretion in MLR was significantly suppressed.

The possible reasons of the inhibited proliferation are manifold. The decreased IL-2 secretion can be the reason and also the consequence of the suppressed lymphocyte activation and proliferation. IL-2 is a necessary growth factor for T cell proliferation after antigen stimulation. It is also imaginable, that treatment with Co-PPIX in MLR and LTT increased the secretion of the antiinflammatory cytokine IL-10, which inhibits the expression of MHCII on APC. It is unlikely, however, that inhibition of lymphocyte proliferation is due to cytotoxic effects of Co-PPIX. So, it has been published that HO-1 induction inhibits apoptosis (Katori et al, 2002; Sass et al, 2003; Wagner et al, 2003), and we did not observe Co-PPIX cytotoxicity in 4 and 24h PBMC culture.

In contrast McDaid and colleagues showed an other key mechanism of HO-1 in immunomodulation. They saw that HO-1 induction (Co-PPIX) mediates activation induced cell death of allo-antigen-responsive murine $CD4^+$ T, which was through the Fas/CD95-FasL signal transduction pathways (McDaid et al, 2005). In turn Zhang and colleagues demonstrated that HO-1 suppression by siRNA in endothelial cells and in mouse lung during I-R injury enhanced apoptosis via increased Fas expression and caspase 3 activity (Zhang et al, 2004). Similar were also showed for Jurkat and primary CD4+ T cells, which are resistant to Fas-mediated apoptosis after HO-1 induction (Choi et al, 2004).

In accordance with our data in PBMC, Yamashida and co-workers showed that addition of biliverdin inhibits IL 2 secretion after T cell stimulation with anti-CD3 and anti-CD28 (Yamashita et al, 2004).

Moreover, Woo and colleagues also demonstrated suppression of alloreactive splenocyte proliferation after Co PPIX treatment of mice, however, did not observe inhibition of IL 2 secretion (Woo et al, 1998). The inhibition of alloreactive lymphocyte proliferation in *in vitro* and *ex vivo* assays corresponds to published protective effects of HO-1 in transplantation models (Woo et al, 2000; Yamashita et al, 2004).

4.4 HO 1 is selectively induced in antigen-presenting cells and inhibits the monocytic expression of accessory molecules and the differentiation and maturation of MDDC

4.4.1 HO-1 induction in APC

Co-PPIX treatment leads to a strong induction of HO-1 expression and activity in human monocytes and monocyte-derived dendritic cells but not in T cells. These results suggest that APC are the main source of enhanced HO-1 activity. This is in agreement with our *ex vivo* data showing stronger MLR inhibition after Co-PPIX treatment of donor mice for the stimulator than responder splenocytes and with recent reports (Kitada et al, 2001; Lee et al, 2002; Philippidis et al, 2004).

Literature data additionally implicate a constitutive expression of HO-1 in CD25-positive T-helper cells and HO-1 induction in CD25-negative T cells after 12 hour stimulation with anti-CD3 and anti-CD28 (Pae et al, 2003). Moreover, in our study the moderate inhibition of *ex vivo* MLR after treatment of the donor mice for the responder splenocytes may also indicate some direct effects of Co-PPIX on T cells.

It is more likely, however, that HO-1 induction in splenic APC exhibits paracrine effects also on T cells by enhancing the CO and bilirubin levels (Pae et al, 2004). In our study bilirubin concentrations were determined in culture supernatants from Co-PPIX-treated T cells, monocytes and monocytic THP-1 cells. Whereas no bilirubin was detected in supernatant of T cell cultures increases were observed in cultures of monocytic cells. Moreover, we observed a systemic increase of bilirubin after *in vivo* treatment of mice as it has also been published by others (Quan et al, 2001; Woo et al, 1998).

4.4.2 Immunophenotypic changes in APC

Based on the strong HO-1 induction and considering the role of antigen-presenting cells for T cell immunity, the effects of Co-PPIX treatment on the immunophenotype of monocytes and the immunophenotypic differentiation and maturation of monocyte-derived dendritic cells were tested.

The expression of MHC class II and accessory molecules is crucial for the antigen-presenting capacity of APC (Janeway et al, 2002). So, the inhibition of monocytic expression of HLA-DR and CD86 by Co-PPIX treatment indicates a direct inhibitory effect of HO-1 induction on APC function which may also contribute to the diminished T cell response which we have observed in immunoassays with human PBMC.

The heterogeneous cell population of Dendritic cells – myeloid (Langerhans cells), plasmacytoid and follicular have distinct immunophenotype, localization, and differentiation status and they are different in functions in the immune system, including the induction of effector immune responses and the establishment of central and peripheral self tolerance (Banchereau et al, 2003; Shortman et al, 2002).

The differentiation of monocytes either towards DC or macrophages represents an essential balance in immune regulation either promoting the initiation of adaptive immunity or the non-immunogeneic uptake and elimination of antigens by scavenger function of macrophages. An example for this antagonistic regulation of monocyte differentiation is given by the immunosuppressive cytokine IL-10. IL-10 enhances the differentiation of monocytes to macrophages while inhibiting the differentiation to dendritic cells (Allavena et al, 1998).

The influence of Co-PPIX on differentiation of myeloid DC was investigated. The GM-CSF/IL-4-driven differentiation of monocytes into immature MDDC was suppressed by HO-1 induction as demonstrated by reduced surface expression of CD1a and increased levels of CD14. These alterations indicate a shift to a “macrophage status” rather than a “dendritic cell status”. The promotion of macrophage differentiation by Co-PPIX is also supported by the induction of phagocytosis / respiratory burst activity in cultured monocytes (see 4.5).

The maturation of DC can be initiated by stimulation of immature DC with LPS. Mature DC are characterized by high expression of CD83 as well as MHC class II and accessory molecules (CD40, CD80, CD86). The maturation of DC is a requirement for a potent and successful initiation of specific immunity. IL-10 prevent the DC maturation and reverts the immature phenotype of DC into macrophages (Allavena et al, 1998).
Co-PPIX treatment during MDDC maturation potently suppressed the increase in HLA-DR, CD86 and CD83 expression, indicating a HO-1-mediated inhibition of DC maturation and suggesting a diminished antigen-presenting capacity. In these studies, Co-PPIX as a well established tool compound known to induce HO-1 expression was used (Pae et al, 2004; Shan et al, 2000; Woo et al, 1998; Yamashita et al, 2004). In order to determine that the induction of HO-1 is indeed responsible for the effects, experiments using HO-1-transduced immature MDDC were performed. Similar to Co-PPIX treatment, adenoviral HO-1 transduction significantly inhibited the maturation of MDDC.

4.5 Enhanced HO-1 expression in APC inhibits antigen presentation and immunostimulatory cytokine production while increasing IL-10 secretion and scavenging functions

4.5.1 Antigen presentation

The observed depression of accessory molecule expression and the inhibition of immunophenotypic maturation of MDDC indicated a diminished antigen-presenting capacity. In fact, both Co-PPIX treatment and adenoviral HO-1 transduction depressed the antigen-presenting activity of MDDC to T-helper cells as shown by decreased proliferation in MLR and LTT and diminished IL-2 secretion. In addition to direct effects of enhanced HO-1 activity on antigen-presenting cells, the inhibition of T cell proliferation in these *in vitro* standard assays for T cell immunity could also be related to enhanced levels and paracrine effects of the heme degradation end-products. So CO has been shown to inhibit T lymphocyte proliferation (Song et al, 2004).

4.5.2 Phagocytosis and respiratory burst activity

Phagocytosis and oxidative burst are important scavenger cell functions involved in the limitation / resolution of inflammation by uptake, killing and degradation of microorganisms but also apoptotic cells without the initiation of a specific immune response.
The effects of HO-1 induction on phagocytosis and oxidative burst activity were investigated by Co-PPIX treatment of monocytes. Interestingly, a dose-dependent increase of these functions was observed which, however, was lost by using the highest dosage of Co-PPIX (50 µmol/L). The increased phagocytosis after Co-PPIX treatment may be related to the observed augmentation of IL-10 secretion (see 4.5.4). So, IL-10 is known to enhance the differentiation of monocytes to macrophages, which represent potent scavenger cells, and to strengthen the phagocytosis (Fortsch et al, 2000; Grutz, 2005)

An increase of phagocytosis capability in states with enhanced HO-1 expression has been described by several groups. So, Omura and colleagues found an induction of phagocytosis in peritoneal macrophages of bach1-deficient mice. Bach1 is a transcriptional repressor of HO-1 (Omura et al, 2005). Furthermore, Hirata et al. demonstrated an increase in HO-1 expression selectively in Schwann cells which differentiate into phagocytozing cells due to sciatic nerve injury in the rat. Schwann cells are neuroglial cells that wrap around axons in the peripheral nervous system forming the myelin sheath, are known to be injury resistant, survive during myelin degeneration and contribute to the axonal regeneration (Hirata et al, 2000).

Finally, a defect of HO-1 in mice and human is also associated with a strongly impaired phagocytosis activity (Poss et al, 1997a; Kawashima et al, 2002; Yachie et al, 1999).

Taken together, an enhanced HO-1 activity seems to inhibit the maturation and antigen-presenting activity of APC while increasing the development of macrophages and scavenger functions. These immunoregulatory activity may be crucial for the physiological role of HO-1 in the limitation / resolution of an inflammation as well as for the immunosuppressive effects of pharmacological HO-1 induction.

4.5.3 Proinflammatory cytokine production

HO-1 induction also alters the cytokine secretion pattern of antigen-presenting cells.
TNF-α is as proximal cytokine of the inflammatory cascade and has various proinflammatory effects. The pathophysiological relevance of TNF-α for T cell-dependent inflammation has been confirmed by successful treatment of type 1 immunopathies with anti-TNF-α antibodies (Crohn's disease, rheumatoid arthritis, psoriasis) (Den Broeder et al, 2002; Feldmann, 2002; Mascheretti et al, 2002; Ogilvie et al, 2001).
HO-1 induction by Co-PPIX inhibited the LPS-stimulated secretion of TNF-α in monocytes and immature MDDC. A parallel decrease of mRNA expression was found in MDDC. Similarly, an inhibited TNF-a production has been described in murine macrophages treated with Co-PPIX (Otterbein et al. 2000; Woo et al, 1998). Moreover, TNF-α secretion is suppressed in HO-1-transduced and in heat shock-treated murine macrophages (Ensor et al, 1994; Li et al, 2001; Otterbein et al, 2000; Petrache 2000; Schon et al, 2005).

IL-12p70 (p40 and p35 chain) is a cytokine which is secreted by activated antigen-presenting cells. It substantially promotes the Th1 differentiation as well as the activation and IFN-γ secretion by type 1 T cells and NK cells. Remarkably, on the other hand IFN-γ is an important activator of APC, e.g. by increasing their MHC class II expression, IL-12 secretion and proinflammatory function (Murphy et al, 2003; Schmitt et al, 1997). This way IL-12 and IFN-γ establish a positive immunostimulatory circuit. IL-23 is a new member of IL-12 family, which shares the p40 subunit and it's IL-12Rß1 receptor with IL-12. The alpha chain of IL-23 is p19 that binds to the IL-23 receptor. IL-23 is a known inducer of the proinflammatory cytokine IL-17 and promotes the differentiation of $Th_{IL\text{-}17}$ cells. The presence of IL-23 and IL-17 have been linked to the induction and maintenance of inflammation in different autoimmune diseases (Gran et al, 2004; Rosmarin et al, 2005).

Antagonization of IL-12p40 by neutralizing antibodies, which target both IL-12 and IL-23, has been successful as an antiinflammatory approach in phase I studies in psoriasis and Crohn's disease (Kauffman et al, 2004; Mannon et al. 2004; Rosmarin et al, 2005). Animal models for autoimmune diseases, e.g. experimental allergic encephalitis (EAE) and arthritis, with targeted disruption of

IL-12p35, IL-23p19 and p40 chains indicate that IL-23, rather than IL-12, might be the pathophysiological most important cytokine (Becher et al, 2002; Brok et al, 2002; Gran et al, 2002; Gran et al, 2004).
In our study Co-PPIX treatment suppressed the LPS-induced secretion of IL-12p70 and IL-12p40 in monocytes and MDDC. Moreover, the mRNA expression of IL-12p35 and IL-12p40 was inhibited. In accordance to this it has recently been shown that heat stress (43°C) suppresses the expression of IL-12p40 mRNA and IL-12p70 protein after LPS stimulation by blocking of IkB-alpha degradation in murine peritoneal macrophages and in RAW264.7 cells (Li et al, 2001).

IL-18 is a proinflammatory cytokine with strong synergism to IL-12 for Th1 generation and function. It's pathophysiological impact has been implicated for psoriasis, rheumatoid arthritis and EAE (Bresnihan et al, 2002; Ito et al, 2003; Ohta et al, 2001). The results of clinical studies with application of IL-18 binding protein (IL-18bp - Tadekinig-α) in patients with rheumatoid arthritis and psoriasis are pending (Serono biotech & beyond).
In our study HO-1 induction by Co-PPIX significantly inhibits the LPS-stimulated IL-18 secretion in monocytes.

In summary, it is likely that the inhibition of proinflammatory cytokine production in APC contributes to the antiinflammatory effects of an enhanced HO-1 activity.

4.5.4 IL-10 secretion

The LPS-induced secretion of IL-10 was significantly enhanced by HO-1 in monocytes and MDDC. The in contrast rather diminished expression of IL-10 mRNA in LPS-stimulated MDDC implicates the involvement of posttranscriptional mechanisms in IL-10 production (Powell et al, 2000).
Our data demonstrating IL-10 induction by the HO-1 inducer Co-PPIX in human monocytes and MDDC are in accordance to reports describing an induction of IL-10 by CO treatment or by heat shock in murine macrophages (Ensor et al, 1994; Raju et al, 1994; Snyder et al, 1992; Yachie et al, 1999). Moreover, exposition of mice to CO leads to enhanced systemic IL-10 levels (Otterbein et al, 2000).

Interestingly, IL-10 has many immunoregulatory effects which are in accordance to our results for HO-1 induction. IL-10 is known to:
Inhibit the MHC class II and accessory molecule expression as well as the differentiation and maturation of APC (Allavena et al, 1998; Corinti et al, 2001; Grutz, 2005),
Inhibit the antigen-presenting capacity of APC (Grutz, 2005; Steinbrink et al, 1997; Tadmori et al, 1994),

Inhibit the proinflammatory and immunostimulatory cytokine production of APC (Fiorentino et al, 1991; Grutz, 2005),
Augment the phagocytosis and respiratory burst activity of monocytes
(Fortsch et al, 2000; Grutz, 2005).

So, the augmented IL-10 production may crucially be involved in the immunological changes observed after HO-1 induction or adenoviral HO-1 transduction of APC.
Remarkably, Inoue et al. demonstrated that HO-1 transduction (which increased IL-10 expression in macrophages) was able to inhibit acute LPS-induced lung injury in wildtype but not in IL-10 knockout mice (Inoue et al, 2001). Some data, however, indicate that IL-10 is not the only player in the antiinflammatory activity of HO-1. So, in contrast to HO-1 induction, IL-10 was found to be not able to suppress the LPS-induced IL-18 production (Zediak et al, 2003). Moreover, despite a diminished alloreactive response a normal contact hypersensitivity response has been found in viral IL-10-transgenic mice (Ding W et al, 2003).

Interestingly, it has been described on the other hand that HO-1 might be involved in the antiinflammatory effects of IL-10. Lee and colleagues described that the inhibition / knockdown of HO-1 leads to a complete suppression of IL-10 effects in murine macrophages (Lee et al, 2002).
Such a possible positive feedback – HO-1 induces IL-10 and IL-10 effects are mediated by HO-1 induction - would represent a positive regulation circuit for the amplification of the antiinflammatory response as it is also known for the proinflammatory response (e.g. positive circuit of IL-12 and IFN-γ production) (Schmitt et al, 1997).
However, other data are contradictory to this assumption. So, Ricchetti et al. could not reproduce an inhibition of IL-10 activity by HO-1 antagonization in human macrophages (Ricchetti et al, 2004). Moreover, HO-1 knockout mice do not share the severe phenotype of IL-10 knockout mice (Inoue et al, 2001).
Taken together, the increase of IL-10 production in APC likely represents a potent immunoregulatory and antiinflammatory principle of enhanced HO-1 activity.

4.6 Conclusion

In conclusion endogenous HO-1 activity limits and pharmacological superinduction of HO-1 suppresses experimental T cell-dependent skin inflammation. A main mechanism for suppression of T cell immunity is the induction of HO-1 in antigen-presenting cells leading to the inhibition of their differentiation, maturation and function. Targeted induction of HO-1 overexpression may represent a new therapeutic strategy for the treatment of T cell-dependent inflammation (Figure 40).

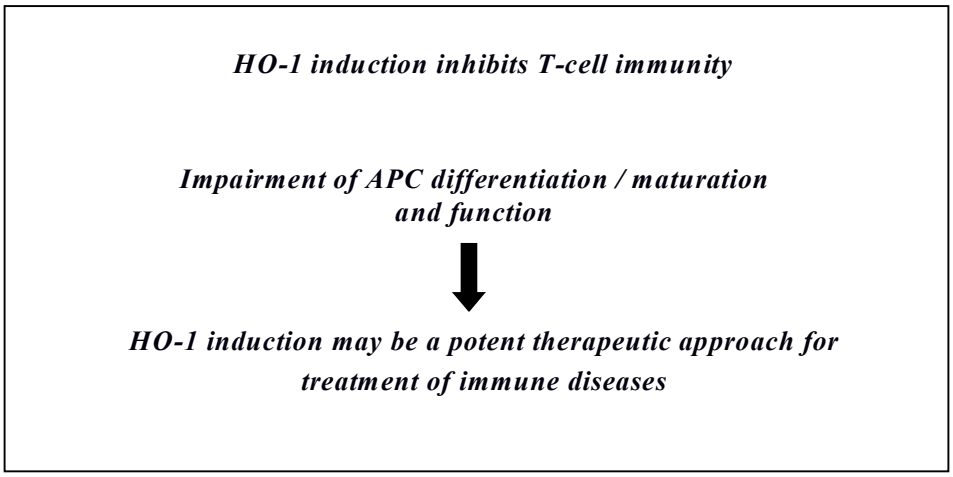


Figure 40. Summing-up
HO-1 induction inhibits T cell immunity. An important mechanism is the impairment of the differentiation, maturation and function of myeloid dendritic cells. Therefore HO-1 may be a potent therapeutic approach for treatment of autoimmune diseases and allotransplant rejection.

5 Summary

Heme oxygenase 1 (HO-1) is an enzyme induced by cellular stress, which catalyzes the degradation of heme to carbon monoxide, ferrous iron and biliverdin / bilirubin. Cytoprotective and antiinflammatory effects of HO-1 induction or transfection as well as for the heme degradations end-products have been reported in several models for inflammation and specific immunity. The HO-1 effects in skin inflammations and the mechanism underlying the antiinflammatory HO-1 properties are poorly characterized.

The aims of this work were :

i. Characterization of HO-1 expression in T cell-dependent skin inflammation both in human diseases and in experimental model
ii. Determination of the physiological role of HO-1 in skin inflammation
iii. Investigation of the effect of pharmacological HO-1 induction on skin inflammation
iv. Investigation of the effects of enhanced HO-1 activity on T cell immunity in *ex vivo* and *in vitro* assays
v. Clarification of mechanisms underlying the inhibition of adaptive immunity by investigation of the effects of enhanced HO-1 activity on the differentiation, maturation and function of antigen-presenting cells.

An endogenous increase of HO-1 expression is found in skin inflammation in psoriasis and in murine T cell-dependent contact hypersensitivity reaction. The main source of the HO-1 protein in inflamed murine skin are infiltrating immune cells. Specific inhibition of HO-1 activity by Sn-PPIX enhances the skin inflammation, demonstrating the physiological importance of HO-1 for the limitation of inflammation. In contrast, induction of HO-1 by the well-known HO-1 inducer, Co-PPIX, reduces skin inflammation both after treatment around sensitization or before challenge. The Co-PPIX effect is abolished by simultaneous application of the HO-1 inhibitor Sn-PPIX.

Skin inflammation (e.g. delayed-type hypersensitivity in mice) is characterized by cell infiltration, plasma extravasation and cytokine secretion in skin. To get evidence about the immunological mechanism of the immunosuppressive effects of Co-PPIX in mice, the alloreactive lymphoproliferative response of spleen cells from Co-PPIX treated mice was analyzed. A strong inhibition of alloreactive proliferation was observed after Co-PPIX treatment of donors, which deliver the stimulator cells (antigen presenting cells). This and the strong HO-1 protein expression in infiltrating, partly MHC class II-

positive immune cells in murine skin were the first indicators for the important role of antigen-presenting cells (APC) for the HO-1 effects on T cell immunity.
In vitro assays in human immune cells revealed that the main source of HO-1 induction by Co-PPIX are antigen-presenting cells, i.e. monocytes, immature and mature monocyte-derived dendritic cells (MDDC) whereas T cells HO-1 expression in T cells was not increased. Similar results have been achieved by measurement of bilirubin as parameter for HO-1 enzymatic activity. These data led to the focus of further investigations on APC.

Monocytes treated with the HO-1 inducer Co-PPIX have reduced expression of HLA-DR and CD86, which play an important role in antigen presentation and the initiation of the adaptive immunity. Additionally the secretion of the proinflammatory cytokines (TNF-α, IL-12p70 and IL-18) is inhibited whereas the potent antiinflammatory cytokine, IL-10, is induced. Moreover, the phagocytosis and respiratory burst activity, representing the scavenger and killing functions of monocytes/macrophages are also induced.

Dendritic cells are highly competent cells for the initiation of adaptive immunity. Induction of HO-1 with Co-PPIX during the IL-4/GM-CSF-driven differentiation of monocyte-derived dendritic cells (MDDC) leads to an impairment of DC differentiation as assessed by diminished CD1a expression with a parallel induction of CD14 as marker for macrophage differentiation. Moreover, both, HO-1 induction by Co-PPIX as well as adenoviral HO-1 transduction, strongly inhibit the maturation of immature MDDC as indicated by suppression of the increase of HLA-DR, CD86, CD83 expression after maturation culture with LPS.
The inhibition of MDDC maturation by HO-1 induction with Co-PPIX or HO-1 transduction has profound functional consequences. So, the DC have a reduced capacity for stimulation of allogeneic and autologous T-helper cell proliferation accompanied with inhibition of IL-2 secretion. Moreover, the LPS-induced expression and secretion of the proinflammatory and immunostimulatory cytokines, TNFα, IL-12p40 and IL-12p70, is diminished whereas the secretion of the antiinflammatory cytokine IL-10 strongly increases.

In summary, the induction of HO-1 is a potent regulator of innate and adaptive immunity with an important mechanism in inhibition of DC maturation and function. These results further elucidate the mechanisms by which an enhanced HO-1 activity suppresses T cell immunity and inflammation. Moreover, our *in vivo* data demonstrate the physiological importance of HO-1 increase for the limitation of T cell-dependent cutaneous inflammation and emphasize that induction of HO-1 represents a potent therapeutic approach for the treatment of skin as well as other inflammations.

6 Outlook

This work characterized for the first time the role of HO-1 in T cell-dependent skin inflammation. Moreover, HO-1 induction in antigen-presenting cells and it's effects on differentiation, maturation and function have been demonstrated as an important mechanism for the antiinflammatory and immunosuppressive activity of HO-1. These results imply the enhancement of HO-1 activity as immunotherapeutic approach for autoimmune disorders and other T cell-dependent diseases.

This study, however, also opens many interesting fields for further investigations:

1) How is the relevance of HO-1 for the activity of established therapies for T cell-dependent skin inflammation ?
   So, HO-1 has been implicated as mediator of the therapeutic activity of UV irradiation (Allanson et al, 2004). Own studies implicate an important role of HO-1 induction for the antiinflammatory effects of fumaric acid esters, an established therapy for psoriasis (Lehmann et al, in Review)
2) Which metabolites (CO, bilirubin) are responsible for the effects of HO-1 induction in inhibition of T cell proliferation, DC differentiation / maturation ?
3) How is the detailed relevance of IL-10 induction for the effects of HO-1 on the maturation and function of dendritic cells ?
4) How are the effects of enhanced HO-1 activity in APC on the Th1 / Th2 differentiation of T-helper cells ?
5) Do dendritic cells with an enhanced HO-1 activity promote the generation of regulatory T cells ?
6) Is HO-1 induction involved in the desensitization of antigen-presenting cells to inflammatory stimuli ?
7) May transfection of antigen-loaded dendritic cells with HO-1 represent a novel approach for the induction of immunotolerance ?
8) May the HO-1 knockdown in dendritic cells represent a novel approach for enhancing the immune response e.g. in vaccination or for treatment of cancer and intracellular infections ?

Taken together, because of the dominant expression and effects of HO-1 in APC and due to the central role of these cells in immunity, all topics which are relevant for T cell immunity may also be interesting for studying the role of HO-1.

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Curriculum vitae

Publications

Joanna J. Listopad, Khusru Asadullah, Claudia, Sievers, Thomas Rittter, Christian Meisel, Robert Sabat, Wolf-Dietrich Döcke.
„Induction of heme oxygenase-1 inhibits dendritic cells and cutaneous T cells immunity“
[in review, Journal of Investigative Dermatology]

Joachim C.U. Lehmann, Joanna J. Listopad, Hartwig Hennekes, Khusru Asadullah, Wolf-Dietrich Döcke.
„Fumaric Acid Diesters induce immunosuppression via Glutathione Depletion and subsequent induction of heme oxygenase 1“
[in review, Journal of Investigative Dermatology]

Further publications in preparation:
Joanna J. Listopad, Khusru Asadullah, Wolf-Dietrich Doecke.
„Characterization of a new IL-2- triggered Th cell population with dominant secretion of IL-17 (Th $_{IL-17}$)“

Berlin, den 17.11.2005

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Oral presentations at congresses

August 2005, 7th World Congress on Inflammation, Melbourne (Australia)
„Characterization of IL-17 secreting T-helper cells (ThIL-17)
Joanna J. Listopad, Khusru Asadullah, Wolf-Dietrich Döcke

Mai 2005, Federation of Clinical Immunology Societies (FOCIS), Boston, (USA),
„HO-1 induction affects the DC maturation and function and adaptive immunity"
Joanna J. Listopad, Thomas Ritter, Robert Sabat, Khusru Asadullah, Wolf-Dietrich Döcke.

September 2004, European Society of Dermatological Research (ESDR), Vienna, (Austria),
„Induction of HO-1 inhibits DC maturation and function and adaptive immunity"
Joanna J. Listopad, Thomas Ritter, Robert Sabat, Khusru Asadullah, Wolf-Dietrich Döcke.

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Poster contributions

Mai 2005, Society of Investigative Dermatology (SID), St. Louis (USA),
„Induction of heme oxygenase-1 (HO-1) inhibits T cell-dependent skin inflammation and depresses dendritic cell maturation and function in vivo"
Joanna J. Listopad, Thomas Ritter, Robert Sabat, Khusru Asadullah, Wolf-Dietrich Döcke

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„Induction of heme oxygenase-1 (HO-1) inhibits dendritic cell maturation and function and adaptive immunity"
Joanna J. Listopad, Thomas Ritter, Robert Sabat, Khusru Asadullah, Wolf-Dietrich Döcke

February 2005, International Research Meeting (IRM), Schering AG, Berlin (Germany),
„Characterization of a new IL-23 triggered Th cell population with dominant secretion of IL-17 (Th $_{IL-17}$)"
Joanna J. Listopad, Thomas Ritter, Robert Sabat, Khusru Asadullah, Wolf-Dietrich Döcke

September 2004, European Society of Dermatological Research (ESDR), Vienna (Austria),
„Induction of HO-1 inhibits DC maturation and function and adaptive immunity"
Joanna J. Listopad, Thomas Ritter, Robert Sabat, Khusru Asadullah, Wolf-Dietrich Döcke

February 2003, Arbeitsgemeinschaft Dermatologische Forschung (ADF), Frankfurt/Main (Germany),
„Immunological characterization and assay development for inducers of the heme oxygenase 1 (HO-1)"
Joanna J. Listopad, Thomas Ritter, Robert Sabat, Khusru Asadullah, Wolf-Dietrich-Döcke

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Joanna Listopad, Karsten Parczyk, Monika Klotz, Robert Sabat, Wolf-Dietrich Döcke

February 2003, International Research Meeting (IRM), Schering AG, Berlin, (Germany),
„Fumaric acid esters (FAE) as approach for therapy in inflammatory skin disease"
Joachim Lehmann, Joanna J. Listopad, Robert Sabat, Hartwig Hennekes, Khusru Asadullah, Wolf-Dietrich Döcke

February 2002, International Research Meeting (IRM), Schering AG, Berlin (Germany),
„Characterization of cytokine modulators in vitro and in experimental models of skin inflammation“
Wolf-Dietrich Döcke, Joanna, J. Listopad, Wolfgang Halfbrodt, Khusru Asadullah, Ulla Mönning

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, die vorliegende Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt zu haben. Ich besitze bisher keinen entsprechenden Doktorgrad und habe mich nicht anderwärts um den akademischen Grad „Doctor rerum naturalium" beworben. Ich erkläre die zur Kenntnisnahme der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät der Humboldt-Universität zu Berlin.

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